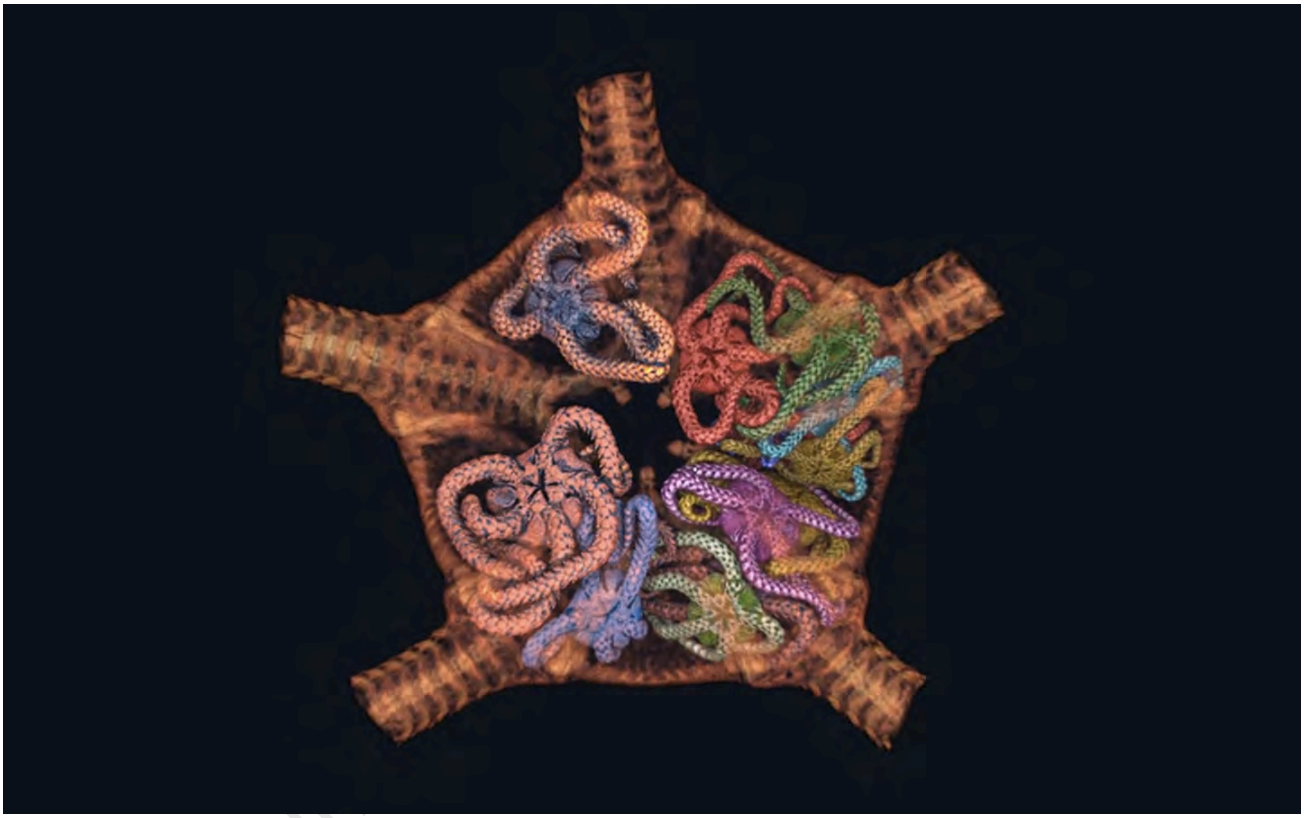
Brooding behaviour in *Ophioderma wahlbergii*, a shallow-water brittle star from South Africa



Minor Dissertation presented in partial fulfilment of the requirements for the degree of Master of Science

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DAAD Deutscher Akademischer Austausch Dienst
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Title image: 3D animated brooding *Ophioderma wahlbergii* from the μCT scan data in Chapter 3, arms cut off. Rendered image is a courtesy of Henry Weber, Volume Graphics GmbH, Heidelberg, Germany.

PLAGIARISM DECLARATION

I know the meaning of plagiarism and declare that all of the work in the dissertation, save for that which is properly acknowledged, is my own.

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ABSTRACT

The brooding behaviour and brooded young are described for *Ophioderma wahlbergii* Müller & Troschel 1842, a large, common brittle star from the coastal waters of South Africa. Twenty specimens were collected each month from June 2013 – May 2014 (n = 240). The species was found to be gonochoric. Females reached maturity at 17 mm disc diameter (dd) and ovaries contained 5(±3) medium to large, yolky eggs with a mean diameter of 0.25(±0.1) mm. The 91 brooding individuals dissected contained an average of 7(±6) and up to 33 young. Besides SEM images to document different developmental stages, micro X-ray computed tomography (µCT) was used to compare brooding of the large *O. wahlbergii* with *Amphipholis squamata* (Delle Chiaje 1828), a tiny brooding hermaphrodite. Brittle stars were scanned with a General Electric Phoenix V|Tome|X NF180 at (30 µm)^3, (9 µm)^3 and (3 µm)^3 voxel resolution and analysed with VGStudio Max 2.2. Brooded young of both species were visualised in 3-dimensional (3D) models, revealing the *in situ* position of juveniles inside brooding ophiuroids for the first time. In addition, the relative volume of the disc occupied by the young was determined. *A. squamata* dedicated 23.31% of its disc volume to brooding and *O. wahlbergii* 14.05%.

At least 50% of female *O. wahlbergii* were brooding throughout the year. No seasonal trends or significant differences between months were found. Individual bursae contained up to eight young at a time and these were mostly of the same stage. Nevertheless, within individual adults, they could be from different clutches. These findings place the species in-between continuous and sequential brooding. No signs of larvae were found, indicating that development is probably direct. Young embryos were loosely attached to the bursal wall and in later stages lay freely inside the bursae. With a maximum of 9.3 mm dd, 38 arm segments and 18.7 mm arm length, these are the largest brooded young known among ophiuroids. Considering the large size of mature embryos, *O. wahlbergii* is likely to be matrotrophic, secreting nutrients through the bursal wall. Small young occurred more frequently than large ones, suggesting that juveniles may emerge at different sizes, or suffer mortality during development. Brooding is a recent discovery in the genus *Ophioderma* and, so far, the only known brooder has a thousand tiny embryos. This study about *O. wahlbergii* shows quite a different reproductive strategy in this genus, with few large eggs developing into the largest known brooded juveniles known in any species of ophiuroid.

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CHAPTER 1.

REPRODUCTIVE TRAITS IN THE CLASS OPHIUROIDEA: A LITERATURE REVIEW

1. Ophiuroid diversity and distribution

The Ophiuroidea, commonly known as brittle stars, basket stars and snake stars, are the most diverse class within the phylum Echinodermata, with 2,139 species and subspecies listed in 16 families and 270 genera (Stöhr et al. 2012; Stöhr et al. 2014b). The name of the group refers to the Greek words *ophis* (snake) and *oura* (tail) because the arms are reminiscent of a snake's tail. Ophiuroids are characterised by their stellate shape with a well-defined central disc, which is clearly separated from generally five long, slender arms (some species have six or more) and they are recorded from all oceans at all depths. There are 201 species recorded in South Africa, of which 152 occur on the shelf (0-200 m) and 21.8% are regarded as endemic to the region (Stöhr et al. 2012).

There is strong support for the monophyly of the Ophiuroidea (Janies 2001). For a long time the orders Euryalida (basket stars and snake stars) and Ophiurida (brittle stars) were treated as sister groups, but their relationship has been questioned (Smith et al. 1995). In fact, the Ophiurida are paraphyletic with respect to the Euryalida (O'Hara et al. 2014).

2. Body structure

2.1. General external characters

Stöhr et al. (2012) provided an illustrated glossary to describe ophiuroid structures. Figure 1 shows a typical ophiuroid body and parts of the arms. Aside from a few species with specialised arm structures, most species look quite alike and have a comparatively simple body organisation that, in comparison to other echinoderms, is fairly consistent. Hyman (1955) reviewed ophiuroid anatomy. She noted that the disc can be either covered with granules or small spines, or can have an aggregation of dorsal plates, which may resemble the juvenile appearance with one central surrounded by other primitive plates. Moreover, the arms are unique among echinoderm classes and show a segmented organisation. The ambulacral ossicles are fused into an internal row

of vertebrae making the arms both flexible and relatively solid. This development eliminated the existence of an ambulacral groove, as found in asteroids (Stöhr et al. 2012). Hyman described the arms as stabilised by oral, dorsal and lateral arm plates, with the latter having 2-15 spines leading to a vertical row (Figure 1), and podia are reduced to a pair of small papillae (or tentacles) per arm joint. The podia are tube feet without suction cups and ampullae. Altered spines, known as tentacle scales, protect them.

On the oral side, Hyman described the arms as embedded into the disc, continuing to the mouth angles. She remarked on flagellated or ciliated areas, mostly on the oral arms that carry on towards the mouth frame, the centre of the oral surface, which is surrounded by five interradial wedge-shaped jaws projecting further inside (Figure 1, B). Leading to each base of the arms there are genital slits, which are supported on each side by an elongated genital shield.

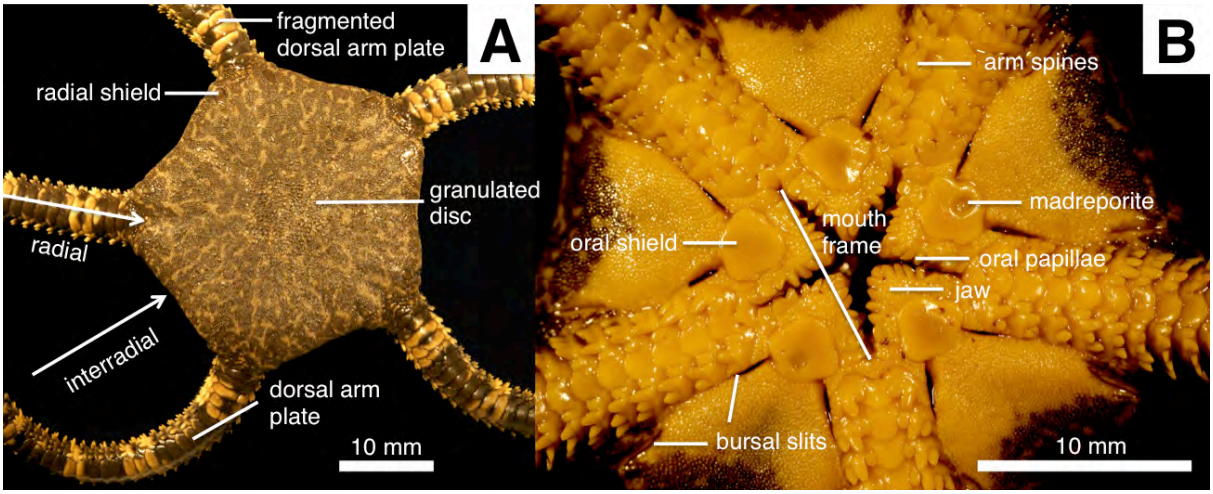

Figure 1. External characters of *Ophioderma wahlbergii*. **A** dorsal (aboral) disc and part of the arms. **B** oral surface of disc with two slits to each bursa.

2.2. Internal anatomy

As mentioned above, ophiuroids have an endoskeleton with deep vertebra-like, fused ossicles providing the structure of the arms and, more superficially, shields stabilising arms and the disc. Hyman (1955) pointed out that each vertebra corresponds to an external arm "joint" covered with one set of arm shields. There are two pairs of intervertebral muscles between successive vertebral ossicles, an upper (dorsal) and a lower (oral) pair, which can be merged into one, but the muscles are generally able to move the arm in any direction. A complex arrangement of concentric muscles operates the jaws or oral plates (Carnevali et al. 1994), which are a homologue to the arm

vertebrae (Stöhr et al. 2012). Food, broken by the jaws, is passed on through the mouth frame to the mouth by the two tube feet closest to the mouth, termed buccal tentacles. Hyman outlined the digestive system of ophiuroids as the easiest organisation possible, where food both enters and leaves the sacciform stomach through the actual mouth, a circular opening in the peristomial membrane. There is no intestine or anus.

The madreporites of brittle stars are hidden (Ferguson 1995). Instead, the water vascular system connects through the axial complex to the surrounding water by one or more hydropores in the oral shields, a part of the mouth skeleton (Figure 1, B). Ophiuroids do not have a centralised nerve system. The system rather consists of a ring nerve in the disc, which sends a radial nerve into each arm, proceeding to its tip. All body parts, including the podia, body wall, mouthparts, stomach and gonads are interlinked to this main system. In an overview of 12 species, sensory receptors were found mostly in the spines and the tube feet (Morgan and Jangoux 2004). Nonetheless, its decentralised architecture and the nerve system, which includes specialised giant motor neurones (Stubbs and Cobb 1981), enables rapid motions, as well as excellent sensory abilities. Respiration occurs through the tube feet, as well as through the bursal wall (Pentreath 1971; Hajduk 1992).

3. Biology

The two general types of ophiuroids are the brittle stars, which have un-branched arms, and the basket stars, which have developed branched arms. With their solid arm structure, ophiuroids use their entire arms, but rarely their tube feet, for locomotion and are therefore the most mobile of all echinoderms (Rowe and Gates 1995). Some bathyal species with less dense ossicles and special long, slender arms or spines are even able to swim (Hendler and Miller 1991). The arms are important tools for acquiring food and ophiuroids display a variety of feeding types. Although most brittle stars are opportunistic feeders that scavenge organic material from the sea floor, some predatory species use their arms to lasso other marine invertebrates (Hyman 1955). Others stick their arms into the current for filter feeding. In fact, the branched arms of basket stars are particularly adapted for filter-feeding purposes (Warner 1982).

Ophiuroids are commonly found in the littoral zone, where they inhabit all types of bottom (Stöhr et al. 2012). Various species live buried in the sand, but most tend to hide beneath rocks (Hyman 1955). When rocks are turned over they quickly return to

their crevices in search for shelter. Their high mobility is one explanation why ophiuroids are so successful, as prey or food can be approached quickly and also agile movements allow an escape from risky environmental conditions (Hyman 1955).

As in other echinoderms, ophiuroids' coelomic fluid is isosmotic to the environment (Binyon 1972), hence is in open contact with the surrounding water (Ferguson 1995). Together with the need to calcify their skeleton this characteristic has led to environmental stressor studies with ophiuroids as model organisms (Walsh et al. 1986; Dupont et al. 2008; Dupont et al. 2010; Wood et al. 2010). In combination with larval development, classical research focused on the description of species new to science and many species were described after extended research expeditions in the late 18th and early 19th century (e.g. Lyman 1882). Brittle stars can have remarkable longevity of more than 30 y (Hendler 1991), and population dynamics are important to understand their ecological role (Packer et al. 1994; Piepenburg and Schmid 1996). In many places, ophiuroids are known to form large aggregations (Broom 1975; O'Connor et al. 1983; Aronson 1989) and are main contributors to biomass (Fujita and Ohta 1989). This has resulted in considerable research into their behaviour and ecology (Warner 1971; Woodley 1975; Warner 1982; Vopel et al. 2003; Morgan and Jangoux 2004; Warner and Woodley 2009). Other studies look at anatomy (Nichols 1972; Cobb and Stubbs 1981; Emson and Wilkie 1982; Ferguson 1995) or combinations of the mentioned topics (Hyman 1955; Clark 1968). A research gap exists in biotechnological applications and echinoderms as a whole, but ophiuroids in particular, are fairly under-investigated in terms of derived molecules, for example as therapeutic treatments (Petzelt 2005). Generally, ophiuroids attract less attention than other echinoderms, probably due to their smaller size and more cryptic habits (Hyman 1955).

In 1975, 55 of the 2000 species known at that time were recorded as viviparous, hence brooding (Hendler 1975). The existence of parental care in this rather less highly-developed group has been the basis for an on-going scientific debate (see Gillespie and McClintock 2007). Besides the developmental mode itself, great emphasis has been placed on understanding selection pressures leading to brooding, especially after the Challenger Expedition (1872-76) had yielded many brooding echinoderms, in particular in the Southern Ocean (see Thomson 1876). Even in recent times new brooding species and developmental strategies are being discovered, indicating that the list of species brooding their young is not yet complete (Hendler 2002; Byrne et al. 2008; Stöhr et al. 2009; Martín-Ledo et al. 2012). This review gives an overview of general aspects of the

Ophiuroidea, but highlights the reproduction and developmental biology, focussing on brooding within the genus *Ophioderma*.

4. Reproductive biology

4.1. Asexual reproduction

Asexual reproduction in ophiuroids occurs by means of fission, the division of one animal into two halves. Subsequently, the two halves regenerate to form two new individuals with the same genes and of the same size (Mladenov and Emson 1984). Technically, but depending on the definition, selfing in hermaphroditic brooding species could be regarded as asexual reproduction. However, because it includes the fusion of sex cells, in this review, hermaphroditic species with the potential to self-fertilize are discussed under sexual reproduction. Parthenogenesis, the development of embryos without fertilization, is rare and hermaphroditic ophiuroids do not show any tendency to reproduce parthenogetically (Boissin et al. 2008b). One of the few brittle stars with a parthenogenetic reproduction is *Ophiomyxa brevirima*, a small brooding species from New Zealand that has separate sexes (Garrett et al. 1997).

Fissiparity is the predominant mode of asexual reproduction in brittle stars (Emson and Wilkie 1980). Mladenov et al. (1983) provided a detailed illustration of the procedure of fission in *Ophiocomella ophiactoides*. About 45 ophiuroid species are known to be capable of reproducing by fission (Mladenov 1996). Nevertheless, brittle stars that divide into two halves are able to reproduce sexually as well (Mladenov and Emson 1984; Chao and Tsai 1995). Generally, fissiparous species have six arms in order to divide in the middle, and are of noticeably small size, with a disc diameter not larger than a few millimetres (Hyman 1955). The latter characteristic of small body size is shared with brooding species, of which many are hermaphrodites. Small body size appears to be correlated with modified reproductive patterns like fissiparity and brooding (Emson et al. 1985, see Section 7.2.).

4.2. Sexual reproduction

4.2.1. Bursae and sexual organs

The genital slits are the openings into the bursae, which are sac-like invaginations into the coelom. Hyman (1955) described the bursae as pouches of inturned body wall with genitorespiratory functions. She highlighted that the bursae are

peculiar to ophiuroids, hence important to their biology, and adapted in many species for respiration and brooding purposes. Due to the presence of these bursae, ophiuroids are thought to be pre-adapted to brooding (Hendler 1979b). In this review, the bursae are of central interest, as they serve as the main brooding structure for embryos.

Usually, there are ten bursal sacs. Deviations can, for example, be seen in *Ophiothrix fragilis*, where the sacs are subdivided into a total of 20 (Smith 1940 as cited in Hyman 1955). A radial coalescing of sacs, i.e. above the arms, is also seen in *Ophiomitrella corynephora* and *Ophiacantha densispina* (five sacs), as well as complete amalgamation into one large sac in *Ophiomitrella hamata* (Mortensen 1933a; Mortensen 1936). Mortensen (1933a) noted that the latter three species are viviparous. The bursal wall and genital slits are usually ciliated, providing a continuous water flow (Hyman 1955; Pentreath 1971). Inside the coelom, the gonads lie in proximity to the bursae. Suspensor cells close to the genital slits connect the sac-like structures to the bursal wall (Buckland-Nicks et al. 1984). Ophiuroid gametes are released into the bursae through a gonoduct (Smith 1940; Buckland-Nicks et al. 1984; Hendler 1991). Hyman (1955) remarked on the existence of species with partially or completely reduced bursae, where the discharged eggs directly enter the surrounding water. She also noted another alteration of the anatomy of the bursal slits in the genus *Ophioderma*. Here, the middle parts of the genital slits are fused together, leading to two separate openings per pouch, one peripheral, close to the arm base, and the other proximal, towards the mouth (Figure 1, B). In this genus, the peripheral slit is incurrent and the proximal one excurrent (Hendler 1991). Nevertheless, like in most species, there are 10 bursae and the gonads of *Ophioderma* are attached to the bursal wall.

4.2.2. Structure and number of gonads

Size, number, colour and organization of gonads differ among species. Generally, the gonads of ophiuroids are spherical or tubular structures. However, the ripe gonads of some species can be branched or lobed and the structure may differ among males and females (Hendler 1991). This gonadal sexual dimorphism can, for example, be seen in *Amphioplus abditus*, where the testes are branched and the ovaries are small sacs (Hendler 1973). Hyman (1955) described a variety of gonad arrangements, from a single or two large gonads attached to each bursa with one on the radial and one on the interradial side, to a number of smaller gonads in various compositions and locations. Later, Hendler (1991) reviewed studies with the number of gonads on the sides of each

bursa for 20 hermaphroditic and 13 gonochoric (only one sex in each individual) species. For the hermaphroditic group, he listed 13 species with mostly one adradial (close to the arm), male gonad and only three species with female gonads lying adradially. He found 11 species with only 1-2 ovaries on the opposite, interradial side of the bursa. In 13 out of 20 species, there were solely female gonads lying on the interradial side, but none of the species had solely male gonads interradially. The remaining species had either both sexes adjoined or ovotestes (both sexes in one gonad), or the gender was unknown. A similar pattern can be seen in the gonochoric group. While there were always 1-8 interradial gonads, eight out of 13 gonochoric species had no gonads adradially. Although the review lacked size information, these findings lead to the presumption that the presence of the arm on the adradial side limits the available space for gonadal growth. Hence, there are more gonads interradially. Furthermore, in hermaphroditic species, there is an increased occurrence of male gonads on the radial side of the bursa. In addition to the location, Hendler (1991) provided another list with the maximum total numbers of gonads per individual for 72 species. Of these, 33 species had 10-20 gonads and 24 species had 50 or more, with a maximum of 500 gonads in *Amphioplus abditus* and 10000 gonads in *Gorgonocephalus eucnemis*. The ophiodermatid *Ophioderma brevispinum* had 400 gonads.

4.2.3. Colour of gonads

The colour of gonads varies from white or grey to yellow, brown and green to bright orange, pink or red (references in Hendler 1991). Hendler listed four species within the genus *Ophioderma* where the gonad colour ranged from blackish, brownish-green to orange-brown. About 10 different colour patterns, from white, brown and green to yellow, are described for the recently-discovered species complex of *Ophioderma longicauda* in the Mediterranean Sea (Stöhr et al. 2009).

4.2.4. Spermatozoa and oocytes

Ophiuroid sperm has the typical sperm structure of a spherical head, a distinct midpiece and a prominent tail. Hermaphroditism and vivipary in the cosmopolitan species *Amphipholis squamata* have not led to adaptations in sperm structure or spermatogenesis (Buckland-Nicks et al. 1984). It seems that brooding or hermaphroditic species, in which modified gametes could be expected, produce sperm of typical form (Hendler 1991).

In contrast, the composition and especially the size of the oocytes increase with the amount of yolk reserves placed into the egg. Kessel (1968) studied the ultrastructure of ophiuroid oocytes and the formation of yolk granules. Byrne (1988; 1989) gave insights into vitellogenesis, the deposition of yolk. In the oocytes of *Ophioderma panamense*, the endoplasmic reticulum and the Golgi complex play an important role in the elaboration of proteid yolk granules (Kessel 1968). Moreover, oocytes of the brooding species *Ophiolepis paucispina* accumulate yolk bodies by sequestering yolk precursors from the haemal fluid (Byrne 1989). The purpose of yolk-reserves is to foster the development of non-feeding larvae. Although egg size can vary intraspecifically and among populations, the size of ripe oocytes allows conclusions to be made about the reproductive strategy and ecology of a species (Hendler 1991). Different larval developmental modes are discussed in more detail in Section 5.1. (also see Figure 2).

In his review on reproduction in Ophiuroidea, Hendler (1991) collected information on the maximum oocyte size of 112 species. According to this, the oocyte diameter in ophiuroids ranges from 0.05 mm in *Ophiactis kroeyeri* to 1.5 mm in *Astrospartus mediterraneus*. However, the second largest oocytes measured were 1 mm in diameter, indicating that the unusually large size in *A. mediterraneus* may be an exception. With 61 out of 112 species with oocytes $\leq$ 0.25 mm, more than half of the ophiuroids had small eggs. Of the 77 brooding species listed, the oocyte sizes were available for 26. Of these, all except six had oocytes $\geq$ 0.3 mm, the small *A. squamata* having the smallest (0.1 mm) and *Astrothorax waitei* the largest (1 mm) diameter of oocytes among brooding species. The egg size of four *Ophioderma* species was given. *O. brevicauda*, *O. brevispina* and *O. cinerea* have a maximum oocyte diameter of approximately 0.3 mm and *O. longicauda* has one of 0.18 mm. However, as mentioned above, *O. longicauda* seems to be a species complex and all records about this species should be handled with caution (see Stöhr et al. 2009; Boissin et al. 2011). In addition, after Hendler's detailed list of records, large oocytes of 0.49 mm have been found in *Ophioderma januarii* (Borges et al. 2009).

The specific gravity of oocytes is an important feature influencing the fertilization success and embryo dispersal (Hendler 1991). Eggs can be negatively, positively or neutrally buoyant. The oocytes of *O. brevispina* and *O. cinerea* sink, but it is important to note that oocyte buoyancy has not been correlated with mode of development (Hendler 1991, and references therein). Furthermore, ophiuroid oocytes are often heavily pigmented (see Section 4.2.3.) and there have been reports of bioluminescent ovaries in

the deep-sea brittle star *Ophiomusium lymani* (Herring 1978). The functional significance of these properties is not resolved (Hendler 1991).

4.2.5. Reproductive and spawning behaviour

All in all, behavioural studies of ophiuroids are rare. In terms of reproductive behaviour, male-female-interactions with the purpose of spawning or pairing are not observed often. Although more interactions are likely, they remain unreported, possibly due to their brevity and timing (Hendler 1991).

4.2.5.1. Pseudocopulation

Pseudocopulation is a copulation-like behaviour that serves a reproductive function. In ophiuroids, it includes the approach of sexual partners with mutual touching of discs and arms. This behaviour brings male and female gametes closer together to increase fertilisation success. Pseudocopulation in ophiuroids has been reported for a few species (Himmelman et al. 2008). Active pairing during the spawning period, but not aggregated spawning, is reported for *Ophiocomina nigra* (Gorzula 1973, as cited in Hendler 1991). In addition, there are several dimorphic ophiuroids where there is a permanent association between a female and one or more males. Mortensen (1933b) discovered that, in these species, the dwarf male often clings inversely to the oral side of the larger female, so that the bursal slits of the pair lie close together. Although there have been misinterpretations with similar associations, this behaviour can be seen as a sexual pairing (Hyman 1955; Hendler 1991; Tominaga et al. 2004), and thus pseudocopulation.

4.2.5.2. Spawning

Hyman (1955) noted a general trend of males initiating spawning of females by the release of their gametes. Indeed, Hendler (1991) confirmed this finding and listed six species (including *O. brevispina*) where males spawn first. However, by referring to numerous exceptions, he argued that male-induced spawning is not necessarily the rule. Nevertheless, females of *Ophioderma rubicunda* and *O. squamosissima* release their eggs 30 min after males start spawning (Hagman and Vize 2003). Moreover, Hendler could not find support for the supposed idea that massive aggregations of ophiuroids are related to reproductive behaviour. The finding that five species of ophiuroids from Australia and New Zealand require an aggregation of males and females to spawn

contradicts this (Selvakumaraswamy and Byrne 2000a). Nevertheless, in the laboratory, Hendler observed the behaviour of successive spawning in *Ophiocoma echinata*. After the male had spawned, the female deposited the eggs and then moved aside directly for the male to crawl over the clutch of eggs and spawn again. This discovery indicates that, although mostly unreported, ophiuroids can have complex reproductive behaviour.

At the time of Hendler's review, Hendler and Meyer (1982) had published the first and only *in situ* observation of ophiuroid spawning behaviour, and found that, in the Palau Islands, Pacific Ocean, *Ophiarthrum pictum* started spawning shortly after sunset and interrupted its spawning when illuminated by diving lights. They noted that, in order to spawn, individuals emerged from their crevices and took up exposed positions on colonies of coral, raised their disc above the substratum, supported by their arms, and expanded and contracted their discs in order to spawn. This observation is in strong agreement with the general understanding of how ophiuroids spawn, and that most of them are nocturnal spawners (Hyman 1955). To reduce the loss of spawning adults and of gametes to visual predators, spawning after dark might be selected for during the evolution of broadcast spawning (Hendler 1991). Simultaneously, it would explain the low number of reported observations. Spawning postures are also widespread in brittle stars and include waving movements of the arms, pumping motions of the disc and lashing of the proximal tube feet to spread the gametes (Hendler 1991). Himmelman et al. (2008) provide a video of a mass-spawning event of echinoderms, including pseudocopulation and spawning postures of ophiuroids, which can be viewed at http://www.int-res.com/articles/suppl/m361p161_app/.

4.2.5.3. Behaviour of brooding species

Unfortunately, there is no information available as to whether brooding species show altered reproductive behaviour. It can only be speculated that a spawning posture of females is unnecessary, because they retain their oocytes internally. Male individuals could still show spawning postures. Because brooding brittle stars need to fertilize their oocytes internally, pseudocopulation might be advantageous. However, most brooders are hermaphrodites, with high selfing rates (see Section 7). It would be interesting to know whether brooding species still have a tendency for nocturnal activities. In the laboratory, juveniles of the brooding species *Amphipholis japonica* emerged over night (Matsumoto 1915).

4.2.6. Spawning and reproductive cycles

Ophiuroids can have either continuous (all year round) or discrete spawning periods, and, within populations, the reproductive cycle can be comparatively synchronized or asynchronous, depending on the proportion of individuals in the identical phase of gametogenesis (Hendler 1991). Hendler (1991) raised the question of whether continuous spawning is a function of individuals having continuous gametogenesis and frequently recurring spawning, or of overlapping, asynchronous reproductive cycles of individuals in the same population. He found evidence for both. Furthermore, he remarked on a third group of asynchronous populations, which spawn throughout the year, but show intensified seasonal peaks. He reviewed the duration of spawning periods for 43 species, in which populations were sampled for at least one year. Of these, nine species bred continuously all year and 12 species had short spawning periods ($\leq$ 3 months). The remaining species either lay in in the middle, or, like seven species, had different lengths of spawning periods depending on the study and/or location. Both discrete and continuous spawning periods are known for *Ophioderma* (Table 1).

4.2.6.1. Seasonality and climate

It is generally assumed that spawning periods get longer towards lower latitudes (Hendler 1979a; Singletary 1980) and that warm-water and deep-sea populations tend to reproduce continuously (Hendler 1991). However, the diverse group of brittle stars exhibits many adaptations and the numerous exceptions make it difficult to delineate a clear rule. Tropical brittle stars can have very brief spawning periods, as in *O. brevicauda* (June-July, Table 1), while long, but discrete, periods of spawning are not uncommon in the tropics (Mladenov 1983). In New South Wales, populations of *Ophionereis schayeri* can spawn for up to eight months with a synchronous peak in summer and asynchronous gamete release throughout winter (Selvakumaraswamy and Byrne 1995). Moreover, spawning seasons can shift from year to year and among populations. Histological observations of the gonads of *Gorgonocephalus caryi* revealed spatial and inter-annual differences in the spawning periods (Patent 1969). A population of the Antarctic brittle star *Ophionotus victoriae* studied for four consecutive years showed clear variations in the yearly reproductive effort, which were probably associated with environmental conditions (Grange et al. 2004). Furthermore, inter-population

differences in spawning are reported for *Ophioderma* on the Caribbean coast (Hendler 1991).

Table 1. Reproductive spawning periods and respective sampling locations of species of *Ophioderma* (modified from Borges et al. 2009). Note that *Ophioderma longicauda* is now regarded as a species complex with discrete populations.

Species	Reproductive Period	Location	Reference
Ophioderma appressa (Say, 1825)	Sep-Nov	Panama	Hendler (1991), Hendler et al. (1995)
Ophioderma brevicauda Lütken, 1856	Aug-Nov	Panama	Hendler (1991)
Ophioderma brevispina (Say, 1825)	Jun-Jul	Massachusetts, USA	Hendler et al (1995), Hendler & Tyler (1986), Hendler (1991)
Ophioderma cinerea Müller & Troschel, 1842	All year round (continuous)	Panama	Hendler (1991), Hendler et al. (1995)
Ophioderma longicauda (Retzius, 1805)	Jul-Aug	Mediterranean Sea	Hendler & Tyler (1986), Hendler (1991)
Ophioderma rubicunda Lütken, 1856	Sep-Nov	Panama	Hendler (1991), Hendler et al. (1995)
Ophioderma januarii Lütken, 1856	All year round (continuous)	São Paolo, Brazil	Borges et al. (2009)
Ophioderma wahlbergii, Müller & Troschel 1842	All year round (continuous)	False Bay, South Africa	This study

4.2.6.2. Predictability

It is likely that the seven species of Hendler's table, which have varying reproductive lengths in different studies, had separate populations, or showed temporal shifts in spawning periods. Predictions on reproductive cycles in ophiuroids are therefore difficult to make. Especially for brooding species, information on breeding variations is scarce. In a temperate climate, peaks of the release of juvenile *A. squamata* are linked to seasonality in reproduction and extended breeding seasons are expected in low latitudes, where seasons and associated factors like light and temperature are less variable (Hendler 1975). The described shifts and variations reveal a problem for reproductive research, because even detailed studies considering full annual cycles may be too short. In addition, exogenous and endogenous factors influencing reproduction are mostly unidentified, but seasonality and associated factors undoubtedly influence reproduction cycles in ophiuroids (Hendler 1979a; Rumrill and Pearse 1985). Therefore, it has been assumed that certain factors are responsible for triggering gametogenesis and spawning, but there is a lack of experimental verification. Hendler (1991) critically discussed factors potentially influencing reproduction. He found that temperature, food availability, currents, light and endocrines could all play an important role in ophiuroid reproduction, but that lunar cycles had little effect. However, he highlighted that

reproduction is likely to be triggered by a whole "suite of mechanisms". The assumption that a combination of several factors is needed to induce spawning is supported by the challenging task of inducing spawning of ophiuroids in the laboratory (see Strathmann and Rumrill 1987; Selvakumaraswamy and Byrne 2000a).

5. Development

Due to their cryptic existence, little is known about the reproduction and development of many ophiuroid species. For example, prior to 1975, the larval developmental mode was only known for 4% of the species (Hendler 1975). Although several studies have increased the knowledge about larval development of several species since then (Mladenov 1979; Hendler 1979b; Hendler 1982; Mladenov 1985; Byrne 1991; Selvakumaraswamy and Byrne 2000a; Selvakumaraswamy and Byrne 2000b; Hendler and Bundrick 2001; Hendler and Tran 2001; Hendler 2002; Tominaga et al. 2004; Selvakumaraswamy and Byrne 2004; Fourgon et al. 2005; Cisternas and Byrne 2005; Emlet 2006; Falkner et al. 2006; Allen and Podolsky 2007; Byrne et al. 2008; Martín-Ledo et al. 2012; Falkner et al. 2013), the number of species with known development is still low, given the high number of brittle star taxa. Larvae of ophiuroids are difficult to identify and the same problem applies to postlarvae, which are only known for about 1.5% of non-brooding ophiuroids (Stöhr 2005). The lack of knowledge about taxa-specific development might also be a result of complex reproductive strategies in a diverse group. The diversity of brittle stars has led to the evolution of a great variety of developmental adaptations and reproduction types, including many species that brood their young inside, or close to their body disc (Hendler 1991).

5.1. Developmental modes

Developmental patterns in marine invertebrates can be separated into different reproductive modes (Thorson 1950; Mileikovsky 1971; Chia 1974). For example, they can be based on the nutrition supply (Byrne and Selvakumaraswamy 2006) and the number of eggs or embryos and the time they are kept internally, or the time they spend in the water column (Levin and Bridges 1995). The question of the origin of the larval form and the developmental mode plays a central role when classifying types of development (McEdward and Janies 1997). Which larva is the ancestral form and which traits selected for the evolution of higher-evolved developmental modes? So far, the focus of echinoderm evolutionary development research has been on echinoids and

asteroids, with a comparative approach of egg sizes and developmental modes (Raff and Byrne 2006). These so-called evo-devo studies reveal two main strategies within these two groups. Those species with eggs ≤200 µm have feeding larvae, and those with eggs ≥300 µm have non-feeding larvae living from egg-reserves. However, this dichotomy is less pronounced in ophiuroids, because many species have eggs of intermediate size-classes (Sewell and Young 1997; Selvakumaraswamy and Byrne 2000a; McEdward and Miner 2001; Falkner et al. 2006; Falkner et al. 2013). Therefore, the classification of larval developmental modes, especially of ophiuroids, can be a complex undertaking. The most comprehensive and important approaches are discussed below.

5.1.1. Classification with more than three developmental modes

Strathmann and Rumrill (1987) explicitly described seven developmental patterns for ophiuroids, differentiating between three different kinds of brooding and including asexual reproduction. The authors prioritized the site of development (planktonic, demersal, benthic, aparental or parental) rather than focussing on the type or duration of development, or the supply of nutrients. To differentiate between types of nutrition supply is also a common approach (Poulin et al. 2002; Gillespie and McClintock 2007). Nutritional modes can be planktotroph, lecithotroph (yolk enriched eggs), matrotroph (from the mother) and osmotroph (by osmosis from the surrounding water, Levin and Bridges 1995). Therefore, classification schemes depend highly on the question of research and the species. Levin and Bridges (1995) mentioned this problem and detected the lack of a single basis of organisation to successfully categorize all modes of development. Further, they found that interests of evolutionary ecology and embryonic development overlap and that, with more species knowledge, more categories are being described. As a solution, Poulin et al. (2001) proposed a combined ecological-multifactor-classification, with three independent two-state characters, namely pelagic/benthic, free/protected and feeding/non-feeding. In a similar multifactor classification, McEdward and Janies (1993) identified eight different patterns for asteroids, which were combined by three characters (habitat, nutrition and development). Surely, these multidimensional categories are more precise and they are valid for echinoderms (Poulin and Féral 1996). However, by definition they are uncomfortably complex.

5.1.2. Classification with three developmental modes

Hendler (1975) suggested three groups to classify developmental modes in ophiuroids: *planktotrophic* developers have a long-lived ophiopluteus larva, *abbreviated* developers have reduced ophioplutei or vitellaria larvae and *direct* developers are either brooding ophiuroids, or have an egg capsule. In his review, Hendler (1991) described the three groups as ecologically distinct, though developmentally and taxonomically fuzzy, and provided a list with each group's characteristics (Table 2). This elegant solution is one of the first specifications for ophiuroid development patterns and is straightforward, but expandable. It is widely used in the literature, probably because it is the most comprehensive classification of ophiuroid development (Jablonski and Lutz 1983; Strathmann and Rumrill 1987; Emlet 1991; Levitan 1993; Selvakumaraswamy and Byrne 2000a; Stöhr 2005; Hoareau et al. 2013). Furthermore, in the order *planktotrophic*, *abbreviated* and *direct development*, the three groups can be seen as an evolutionary progression towards parental brooding (Figure 2, Hendler 1991). In the following, the three categories are further described and used as the central terms for reproductive developmental strategies in ophiuroids. Asexual development by fissipary occurs in ophiuroids (see Section 4.1.), but is not considered in this classification of developmental patterns.

Table 2. Characteristics of the major developmental patterns of ophiuroids (modified from Hender 1991).

	Planktotrophic development	***Abbreviated development***	***Direct development***
Maximum range of oocyte diameter (mm)	0.07-0.17	0.13-0.35	0.1-1.0
Maximum number of oocytes per individual	240,000-2,600,000	4,000-30,000	10-2,000
Maximum total volume of oocytes per individual (mm^3)	35.0-697	45.2-135.0	0.1-2,3
Post-larval disc diameter (mm)	0.2-0.9	0.2-0.5	0.6-9.3
Number of post-larval arm segments	0-8	0-1	8-40
Fertilization to completion of metamorphosis (days)	19-216	3-5	unknown
Hermaphroditism	never	never	sometimes

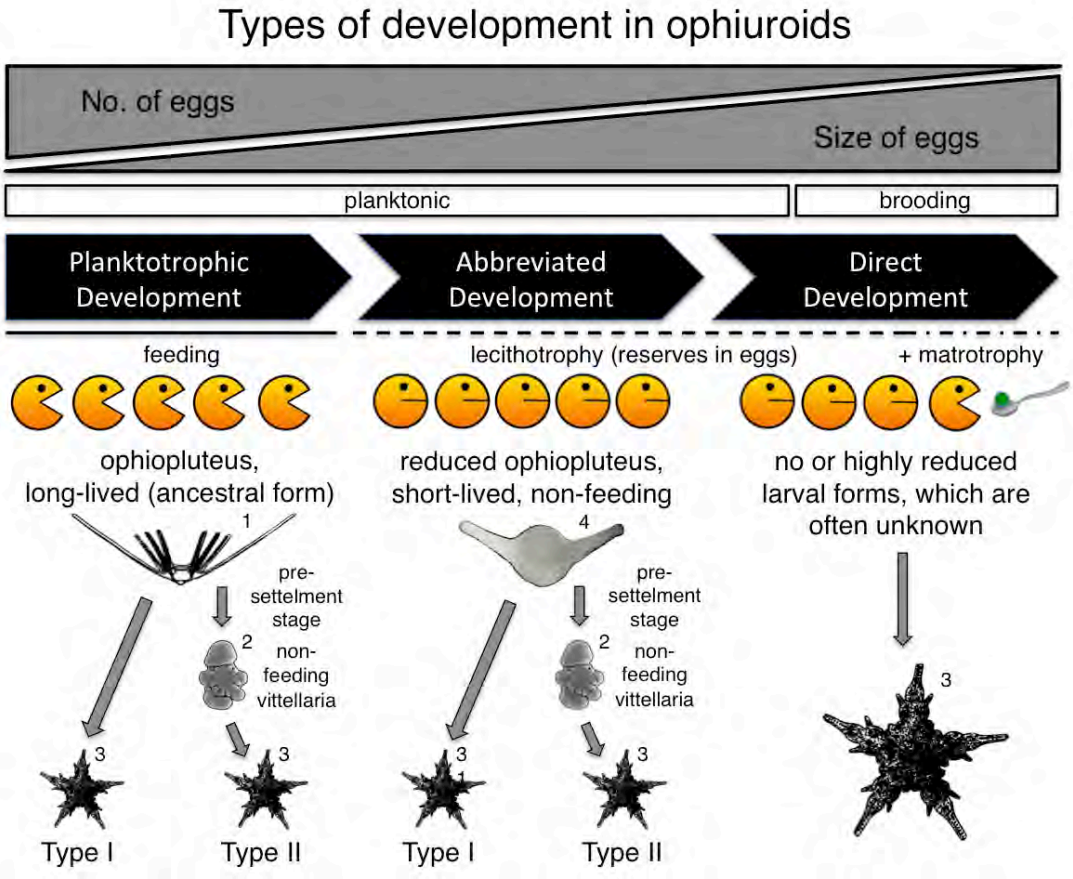

Figure 2. The three central developmental patterns of ophiuroids, reproduction strategies, types of development and the evolutionary progression from planktonic feeding to brooding. [1] ophiopluteus, redrawn after Mortensen (1913), [2] vitellaria, redrawn after Fourgon et al. (2005), [3] young ophiuroid, redrawn after Stöhr et al. (2012), [4] reduced ophiopluteus, redrawn after (Mladenov 1979).

5.1.1.1. Planktotrophic developing larvae

Planktotrophic developing larvae rely on external food sources, develop freely and feed independently in the water column (Levin and Bridges 1995). In broadcasting events, hundreds of thousands of small eggs (70-200 µm) are shed, externally fertilized and then develop into ophiopluteus larvae (Strathmann and Rumrill 1987). The pelagic period of planktotrophic larvae is long, ranging from two weeks to three months (Hendler 1975; Strathmann 1978c; Mladenov 1985). Because the embryo undergoes a larval transformation, development is considered to be indirect. A swimming ophiopluteus with four arm pairs is the general larval form of ophiuroids and the feeding form in planktotrophic development (Hyman 1955, Figure 3, A). Metamorphosis can either occur by a gradual resorption of all larval arms into the body, or only the inner arms are resorbed and the adult rudiment forms between the outer, posterolateral pair, which is then entirely discarded at a later stage (Hendler 1991). The retaining

ciliated posterolateral arms support the transforming brittle star, but also enhance the ability to swim during a brief pre-settlement exploration (Tominaga et al. 2004).

Although the ophiopluteus has the typical form of planktotrophic developers, two types of development exist in planktotrophic species (Figure 2). Type I developing ophiuroids have only a single larval form, the ophiopluteus. Once the Type I ophiopluteus has gained sufficient energy, it metamorphoses directly into the juvenile, whereas Type II developers have two consecutive larval forms, the ophiopluteus and the vitellaria (Selvakumaraswamy and Byrne 2004). Fell (1945) introduced the term vitellaria for a barrel-shaped larva, which also occurs in crinoids and holothuroids (where it is also called doliolaria). Since then, both terms have been used for ophiuroid studies, but the term vitellaria is more common (Hendler 1982). The vitellaria is the secondary stage of Type II developers and in addition to the barrel shape, vitellariae are also characterized by their transverse ciliary bands (Figure 3, B, Grave 1903). In Type II developing species, the non-feeding vitellaria is considered to function in pre-settlement exploration of the substrate (Strathmann 1978a; Mladenov 1985). Nevertheless, the vitellaria form is an exception for planktotrophic developing larvae.

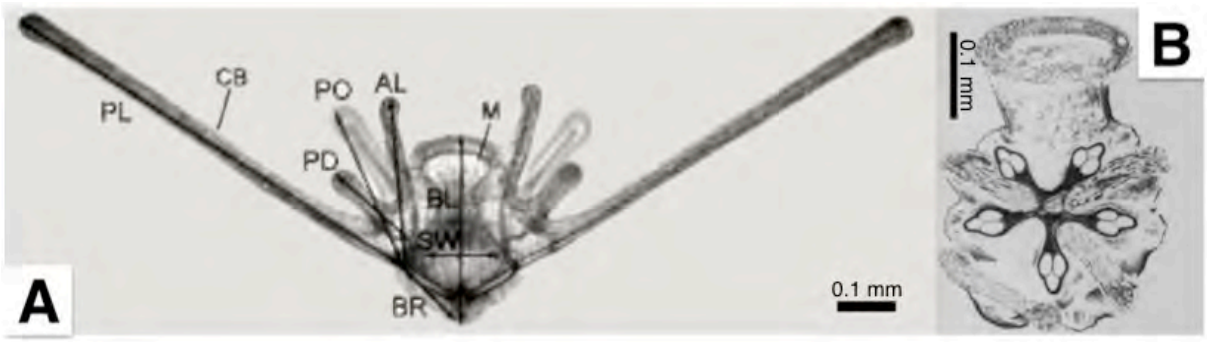

Figure 3. The main larval forms of ophiuroids. **A** Ophiopluteus of *Macrophiothrix longipeda*; *PL* posterolateral arm, *AL* anterolateral arm, *PO* postoral arm, *PD* posterodorsal arm, *BR* body rod, *BL* body length, *SW* stomach width, *M* mouth, *CB* ciliated band, modified after Allen and Podolsky (2007). **B** vittelaria of *Ophionereis annulata*; from Hendler (1982).

5.1.1.2. Larvae with abbreviated development

The remaining two groups are not as clearly distinct as the planktotroph group. In *abbreviated* development, free-living, reduced larvae forms are likely to be lecithotroph (nutrition by yolk, Hendler 1975; Hendler 1991). Here, several thousand medium-sized eggs (139-420 µm) develop into non-feeding larvae and the duration of development in the water column is one week or less (Strathmann and Rumrill 1987, Table 2). Larvae grow by the utilization of reserves (yolk and lipids, lecithotrophy),

which are placed into the egg during oogenesis (Levin and Bridges 1995). Equipped with stored energy, the abbreviated, developing embryo drastically reduces the time before metamorphosis and therefore the time spent in the water column (Table 2). Ophiuroids with abbreviated development are not an embryologically or systematically homogeneous group (Hendler 1975). Instead, they have modified larvae with various characteristics. For example, the ophioplutei can be reduced to a non-feeding two-armed ophiopluteus (Mladenov 1979). Reduced ophioplutei are varied in form and show a rapid development, like vitellariae (Hendler 1991). However, planktonic-lecithotrophic Type I developers with reduced ophioplutei are rare, and for abbreviated development Type II developing species with vitellariae are the general form (Selvakumaraswamy and Byrne 2004).

Because of their distinct appearance, vitellariae in ophiuroids were believed to have evolved secondarily (Williams and Anderson 1975). Most of them lack feeding structures and the continuous ciliated bands around the round body are used solely for locomotion, not for feeding (Hendler 1982). Hyman (1955) perceived this larval form as an aborted pluteus. Indeed, the ophiuroid vitellariae are reduced ophioplutei (Mortensen 1921; Mortensen 1938; Hendler 1982; Selvakumaraswamy and Byrne 2004). In *Ophioderma*, vitellaria larvae with abbreviated development have been described for *O. brevispina*, *O. cinerea* and *O. longicauda* (Grave 1900; Hendler 1975; Hendler 1979a). Hendler and Littman (1986) mentioned eight species of *Ophioderma* with lecithotrophic development, where these three species were included. By interpreting the gametes, Borges et al. (2009) extended the list by including *O. januarii*, although the larvae of this species remain unreported. It seems that ophiodermatids have a clear tendency for lecithotrophy and abbreviated development (Cisternas and Byrne 2005). So far, all investigated species of *Ophioderma* can be placed into this group of larval development.

An unusual demersal, abbreviated development is recorded for *Gorgonocephalus eucnemis* (formerly *G. caryi*), which has a unique irregular, bilateral larva (Patent 1970a). The species *Amphioplus abditus* and possibly *Ophiophragmus wurdemani* bypass the pelagic period by a demersal fertilization membrane where the pluteus-like larval form is ciliated and possesses a paired skeleton (Hendler 1973; Hendler 1975).

In conclusion, abbreviated development is generally lecithotrophic, mostly pelagic, but sometimes overlaps with the other categories. Nevertheless, in comparison to planktotrophy with an ophiopluteus, offspring of species showing lecithotrophy use

the provided energy to reduce both the time in the water column and the developmental time. Although the abbreviated development is diverse, members of this group can be described as ecological analogues (Hendler 1975). Accordingly, the overlap between groups makes classification difficult, but the overlap is a strong argument for a range of evolutionary adaptations and shows a tendency towards reduced pelagic larval phases. This reduction is further increased towards the category of direct development.

5.1.1.3. Larvae with direct development

Direct development is generally used to describe a developmental pattern of larvae that have lost virtually all larvae features (Emlet 2006). Among the three described modes the least information is available about direct development. The first reason for this seems obvious. Direct development is mostly associated with brooding, but conducting *in vitro* developmental studies, as commonly undertaken in planktonic larval forms, is difficult, if not impossible, for embryos that grow under the protection of the adult. The adaptation of brooding might have led to embryos that depend on their parents as protective environment and food source. Even if living embryos can be retrieved from inside the adult, they normally die, as the right condition cannot be simulated. The only example in which embryos were successfully kept alive with artificial food is *Amphipholis squamata* (Fell 1940). However, it is still the case that the earliest, most pivotal, life stages are difficult to rear in the laboratory, another reason being that the number of eggs is generally low (Figure 2). Therefore, the real developmental mode of most brooding species is unknown, but usually it is considered to be direct (Hendler 1991). As larvae are adaptations for an independent life style, larval stages seem unnecessary in the brooded environment. Nonetheless, direct developers might not be as widespread as initially thought. Rudiments of larval forms are reported for a number of brooding species and their status of a direct developer is arguable (Byrne et al. 2008). It is possible that nearly all brooding species have some remnants of larval structures, although at least 70 brooding species are thought to have none and therefore to be direct developers (Selvakumaraswamy and Byrne 2000b). External, non-brooded direct development occurs in an unidentified species named "Kirk's ophiuroid" as well as in the extraordinary species *Amphiodia occidentalis* (Fell 1941; Emlet 2006). In both species, the embryos are individually encased in a tough fertilization envelope, which protects the developing embryo. Normally, the demersal egg capsule is attached to the substratum, but it can be suspended in the water column.

In conclusion, pervasive direct development in brooding ophiuroids is not verified, instead embryos of brooding species have been found to show vestiges of larval characteristics. However, larval forms become dispensable in brooding species, so that brooding species are expected to have highly reduced and modified larvae (Hyman 1955; Hendler 1991). In this discussion, like in Emlet (2006), direct development is considered to be at the extreme end of a morphological continuum of abbreviated development, implying that direct development is the main mode in brooding species. In addition, brooding species follow the general trend of reducing the number and increasing the size of the eggs (Figure 2). Brittle stars that bear live young only shed a few to a thousand large eggs (>300 μm) into the bursae (Strathmann and Rumrill 1987).

6. Brooding

6.1. Vivipary vs. brooding

In ophiuroids there are different ways of brooding, which include external brooding and ovovivipary. In the latter, embryos develop internally, but inside eggs and newly hatched juveniles are directly released through the bursal slit. The term "vivipary" describes trophic interactions between the parent and the young. However, it has been indiscriminately utilized for internal brooding, even when the active transfer of nutrients was not proven, but rather inferred from the growth of large, brooded embryos (Hendler 1991). Like in Hendler (1991), in this review species are termed "brooding" when the transfer of nutrition is not demonstrated.

6.2. External brooding

The broadest definition of brooding may be that the embryos develop in proximity of the adult and gain environmental protection or nutrition, or both, from their parents. In external brooding, the embryos develop beneath, or on the body of, the parent (Strathmann and Rumrill 1987). However, the observation of young individuals attached to larger animals has caused confusion. It has been questioned whether the aggregation is coincidental, whether there might be other reasons, or whether it is truly for the purpose of parental care (Mortensen 1920). The phenomenon of clinging onto adults has been identified for several species, for example *Ophiactis kroeyeri* and *O. asperula* (Ludwig 1898), *Ophiothrix fragilis* (Warner 1971) and *Ophiocoma scolopendrina* (Hendler et al. 1999). In the case of *Ophiothrix fragilis*, 5% of the attached

juveniles are also found inside the bursae of larger specimen, but most are feeding between the arm spines and on the disc (Morgan and Jangoux 2004). Early developmental stages of the basket star *Gorgonocephalus eucnemis* are associated with soft corals of the genus *Gersemia,* and later stages re-attach to adult *G. eucnemis* until they are large enough to start their independent life (Patent 1970b). In all these cases, the behaviour in search of protection and dispersal takes place only after young and adult had been separated. Therefore, the adult is unlikely to be the direct parent, and such an observation cannot be interpreted as brooding (Mortensen 1920).

True external brooding is rare, but can be seen in the tiny species of *Ophyophycis gracilis*, which broods its young beneath the disc (Mortensen 1933a; Mortensen 1933c) and in *Astrothorax waitei* where the young are kept on the oral arm surface (Fell 1952, as cited in Hendler 1975). It is argued that the eggs (0.15-.20 mm) of the minute *O. gracilis* may be too large for internal brooding (Hendler 1975).

6.3. Babysitting brittle stars?

Juveniles of *Ophiomastix annulosa* live on top of the adults of *Ophiocoma scolopendrina* and small individuals also occupy the bursae of the host (Hendler et al. 1999). This behaviour was referred to as "babysitting", but is actually a form of brood parasitism, as know from other animals, such as birds - undoubtedly a remarkable adaptation for an invertebrate species.

6.4. Internal brooding

Hendler (1991), provided a list of 68 ophiuroids, which have been suggested to be internal brooders, but he argued that some species of the list need re-examination. A few recently-discovered brooders must be added, so that the current number probably lies between 70 and 80 species, plus the few external brooders (Selvakumaraswamy and Byrne 2000b; Hendler 2002; Byrne et al. 2008; Martín-Ledo et al. 2012). More as a side-note, even in bursal brooding it may to some extent be questionable whether the bursae can be entirely classified as "inside", as they contain sea water and are invaginations of the oral wall (see Section 4.2.1.). However, true intraovarian brooding exists too. Hendler (1991) found reports on 10 intraovarian brooders and could confirm this for four species, including *Amphiura monorima*, which lacks bursae. The brooding population of *Ophioderma longicauda* in the Eastern Mediterrenean must be added to the list of intraovarian brooding species (Stöhr et al. 2009). Hendler further argued that

the oocytes of internal brooders are probably shed into the bursae and fertilized inside, so that in species that do not self-fertilize, sperm must enter the bursae through the genital slits. In intraovarian brooding, sperm must somehow reach the ovaries, but this is all based on speculations. Hendler, p. 472 stated, "…, we know nothing about the reproductive behavior of the majority of brooding ophiuroids, …".

6.4.1. Embryonic nutrition

Aside from some special cases of intraovarian brooders, internal brooding ophiuroids retain their eggs in their bursae. Most of them appear to be ovoviparous, where the development of the young is completely supported by the nutrient reserves from within the oocyte (Hendler 1979b; Strathmann and Strathmann 1982; Byrne 1989). Nonetheless, Fell (1946) proved the only known embryonic attachment so far in *Amphipholis squamata*, but showed its insignificance with regard to the nutrition supply. Instead, he observed altered bursal walls with "vascular organs or sinuses", when the respective bursa contained one or more embryos, and argued that the bursal wall served as a nutritive organ. Later, Fontaine and Chia (1968) labelled nutrient molecules, which accumulated in larger embryos particularly, and concluded that the young take up dissolved organic material (DOM) through their epidermis, a concept that is widely accepted for planktonic echinoderm larvae (see Manahan et al. 1983). Walker and Lesser (1989) found endosymbiotic bacteria populating the bursal wall of *A. squamata* and suggested that they serve as food, or provide nutrients, to the developing embryos. Ultimately, Hendler and Tran (2001) found the embryos of the deep-sea ophiuroid *Amphiura carchara* pressed against the wall of the bursa. This behaviour is supposedly shown to facilitate nutrient uptake, which is provided from the mother's bursa. Thus, there is substantial evidence that broodcare in ophiuroids includes trophic transfer to the offspring, and at least *A. squamata* seems to be truly viviparous (Hendler 1991). However, for most species, the question of nutritional supply remains insufficiently answered. New insights to this intriguing question can perhaps be found through histological studies of the bursal wall, or more detailed studies on the embryonic position inside the bursae. In particular, species that are brooding very large young require maternally supplied energy for their rapid and continuous growth (Hendler 1975). Nurse eggs or adelphophagy, the consumption of other embryos, were suggested, but are not a sufficient source of energy (Turner and Dearborn 1979).

Table 3. Size of the young of brooding ophiuroids (modified from Hendler 1991, based on the largest embryos reported for each species, dd = disc diameter, No Seg = number of segments, Al = arm length).

Species		dd (mm)	No Seg	Al (mm)	Reference
Amphiophiura	*pachyplax*	0.9	5		Litvinova (1971)***
	sculpta	1.1	8		Murakami (1941)
Amphipholis	*japonica*	1.4	22		Murakami (1940)
	*squamata**	1.2	13	2.2	Ludwig (1899)***, Clark (1914)***, Bernasconi (1926)***, Hendler (1975)
Amphiura	*capensis**	2	20		Djakonov (1914)
	carchara	1.3	4		Hendler & Tran (2001)
	magellanica	1.5	20	3.3	Ludwig (1899)***
	stimpsoni		10		Mortensen (1921)
Astrochlamys	*sol*	2	9		Madsen (1967)***
Astrothorax	*waitei*	2			Baker (1980)
Astrotoma	*agassizii*	1	5	1.7	Bernasconi (1965)***
Ophiacantha	*vivipara**	2.5			Thomson (1878)***, Ludwig (1899)***
	wolfarntzi	1.5			Martín-Ledo et al. (2012)
Ophioceres	*incipiens*	1.5	6		Mortensen (1936)
Ophioderma	*longicauda*	0.8	3		Stöhr et al. (2009)
	*wahlbergii**	9.3	38	18.7	This study
Ophiolebella	*biscutifera*	2			Mortensen (1936)
Ophiolepis	*kieri*		2		Hendler (1979b)
	paucispina		3		Hendler (1979b)
Ophiomitrella	*conferta*		12		Mortensen (1936)
Ophiomyxa	*flaccida*		6		Hendler (1979b)
	vivipara	5	30	20	Studer (1876)***, Ludwig (1898), Mortensen (1933a)
Ophionotus	*hexactis**	8.3	40	20	Studer (1883)***, Mortensen (1921), Morison (1979)***
Ophiopeza	*spinosa*	0.3**	2		Byrne et al. (2008)
Ophioplinthus	*gelida*	0.68	2		Alarcon (1967)***
Ophiozonella	*falklandica*	1.1**	3		Mortensen (1936)

* occur in South Africa, ** estimated from an illustration, *** cited after Hendler (1991)

6.4.2. Size of brooded young

Hendler (1991) reported the known sizes of the embryos of 21 brooding species. Generally, the maximum disc size of brooded young is 1-2 mm (Table 3). Before this study about *Ophioderma wahlbergii*, the largest known embryos, with a disc diameter of 8.3 mm, belonged to the Antarctic intraovarian brooder *Ophionotus hexactis* (Morison 1979, as cited in Hendler 1991). In fact, the enormous size led Turner and Dearborn (1979) to the speculation that the largest young were unable to exit the parent's bursae. This is doubtable, but the mechanisms of how the young exit through a tiny bursal slit have been questioned long ago (Mortensen 1921). Assumptions, e.g. that the adult's disc is autotomized or damaged, are considered disproved. Instead, it has been proposed that mutable collagenous tissue (MCT) plays an important role (Hendler 1991). MCT is unique to echinoderms, can undergo rapid (<1 s), but reversible changes in passive mechanical properties that are under the control of the nervous system and coordinated with the muscles (Wilkie 2005; Ribeiro et al. 2011). No study has looked at the specific MCT characteristics involved in widening and minimizing the opening of the bursal slit. However, given that MCT is involved, it would have the ability to actively stiffen or

soften the tissue of the bursal slit within seconds. Therefore, the biomechanics of MCT are likely to hold the answer of how juveniles emerge from the mother's bursae.

6.4.3. Brooding cycles

Hendler (1991) described two different types of brooding - *sequential brooders* that produce consecutive cohorts of embryos, or *simultaneous brooders* that brood more than one age class of embryos at the same time. He identified 15 species of the former and 12 of the latter group, and *A. squamata* and *O. hexactis* belong to the simultaneous brooders. It follows that sequential brooders tend to give birth discretely and discontinuously, whereas simultaneous brooders may bear young continuously. For example, *Ophionereis vivipara* has a variety of developmental stages in each bursa and probably spawns continuously (Mortensen 1933b). In contrast, *Ophiotjalfa vivipara* broods different age classes in separate bursae (Mortensen 1933d). Furthermore, Hendler pointed out that the gonads of a single bursa can be synchronized within an individual, or can show specific, but asynchronous, gametogenic cycles. No preference for either of the modes was found in warm or in cold water, nor in closely related species, and there is no information on whether brooding species show a general trend towards longer spawning seasons (Hyman 1955; Hendler 1991). Nevertheless, they may have extended spawning seasons and a variety of brooding species brood the entire year (Hendler 1991). Simultaneous brooders with year round brooding can show seasonal peaks in juvenile release. For example, *O. hexactis* releases most of its young during the warmest season (Morison 1979, as cited in Hendler 1991) and *A. squamata* has periods with increased spawning with two distinct cohorts of offspring each year in Connecticut (Hendler 1975).

6.4.4. Number of brooded young

The maximum number of embryos brooded per individual generally ranges from only 1-3 in some species to 200 embryos in *Astrochlamys bruneus* (Hendler 1991). Obviously, there is a space limitation, so that species brooding large offspring may accommodate only a few of these. *O. hexactis* typically has 12-28, and sometimes as many as 86, embryos (Morison 1979, as cited in Hendler 1991). *A. squamata*, with large embryos compared to the small body size, can carry up to 25 individuals (Fell 1946; Hendler 1975). Species with smaller juveniles, i.e. juveniles in less advanced stages, are known to brood up to several thousand yolky embryos. Examples are *Ophioplocus*

esmarki (2,600 embryos, Rumrill and Pearse 1985) or the brooding population of *O. longicauda* (1,048 embryos, up to 110 per bursa, Stöhr et al. 2009).

6.4.5. Duration of brooding

It is difficult to assess the time the juveniles are kept inside the adult before they are released. Based on two distinct cohorts, Hendler (1975) calculated the time that young required to grow from fertilisation to birth at 3-6 months (see also Hyman 1955; Rumrill and Pearse 1985). Embryos of *Stegophiura nodosa* need 4-5 months (Kaufmann 1974, as cited in Hendler 1991) and of *O. esmarki* six month before they leave the bursa (Hyman 1955). The majority of brooding ophiuroids give birth to small juveniles (Table 3). Species with larger juveniles might brood their young for a surprisingly long time. Juveniles of the Antarctic *O. hexactis* appear to have a brooded developmental period of 2-3 y (Morison 1979, as cited in Hendler 1991). Although information on the developmental time is only known for a few species, there is sufficient evidence that it takes at least several months from fertilization until a young offspring is released. It can also be inferred that the larger the embryos grow inside, the fewer individuals can be brooded and the longer they develop inside the brooded environment.

7. Brooding and evolutionary aspects

The three types of reproduction in ophiuroids are planktotrophic, abbreviated and direct development. Evolution is associated with the reduction of egg numbers, the increase of egg size and the acceleration of development, which leads to the complete loss of larval structures in brooding species (Figure 2). Despite variable egg sizes and irregular larvae, comparative evo-devo studies within clades have verified this general principle (Selvakumaraswamy and Byrne 2000a; Selvakumaraswamy and Byrne 2004; Fourgon et al. 2005; Byrne and Selvakumaraswamy 2006; Falkner et al. 2006; Allen and Podolsky 2007; Falkner et al. 2013). But what is the cause of the adaptations leading to this trend in ophiuroid reproduction and what are the reasons for brooding?

7.1. Larval mortality and parental care

All three reproductive strategies have trade-offs influencing the potential reproductive success, and the factors involve parental investment, fecundity, offspring mortality and dispersal, as well as developmental time (Gillespie and McClintock 2007).

Planktotrophic larvae have an advantage of high dispersal, but the disadvantage of high mortality rates (McEdward and Miner 2001). Abbreviated, lecithotrophic developers maintain the advantage of pelagic dispersal, but invest into a larger egg size, which presumably reduces mortality during the risky period in the water column (Strathmann 1978b; Levin and Bridges 1995; McEdward and Janies 1997). On the other hand, species with parental care have higher costs in energy per egg and fewer offspring, hence lower fecundity (Levitan 2000). Although reduced when the development is abbreviated, a major disadvantage of lecithotrophic, pelagic larvae seems to be the dispersal away from favourable parental habitats (Pechenik 1999). Therefore, differences in larval mortality balance parental investment (Strathmann 1985), including brooding as an extreme form of parental care. Consequently, by protecting the brood, minimal offspring mortality in brooding equals out the disadvantages of brooding, which are low fecundity, limited propagule dispersal and boosted parental investment. Altogether, because large propagule size evolves in response to relative survivorship, brooding results in larger young compared to related species that do not brood, and hence, large juveniles are more likely to reach sexual maturity (Shine 1978).

7.2. Adult size and hermaphroditism

The size of the parents plays an important role in the evolution of brooding (Strathmann and Strathmann 1982). Brooding is presumed to be a co-adaptation to competition-induced small size, because small broadcasting species fail to produce sufficient eggs to compensate for losing the majority of pelagic larvae (Menge 1975). Indeed, most brooding brittle stars are of exceptionally small size (Hendler 1991). Additionally, large species with high fecundities may fail to hold and ventilate all brooded embryos they can produce (Strathmann 1985). Hermaphroditism is another striking feature of small brooding ophiuroids, yet its real evolutionary advantage remains unverified (Mortensen 1920; Hyman 1955; Strathmann and Strathmann 1984; Hendler 1991; Leonard 2006). The most plausible speculation is that small brooding species with a limited dispersal range benefit from self-fertilisation. This is supported by the evidence of high selfing rates in hermaphroditic species (Poulin et al. 1999; Boissin et al. 2008a; Boissin et al. 2008b).

7.3. Brooding – a reproduction strategy in cold waters?

In the past, the question of whether brooding in echinoderms is an Antarctic phenomenon has been the basis for an intense scientific debate. Reviews on this topic show that, although there are many brooding species in the Antarctic, this reproduction type is not confined to the cold polar conditions, nor to the deep sea (Pearse 1994; Poulin and Féral 1996; Poulin et al. 2002; Pearse and Lockhart 2004). Instead, there seems to be a variety of conditions selecting for brooding. The most general approach is that 'stress', defined as anything that hinders the animal from successful reproduction, leads to the evolution of brooding (Lawrence and Herrera 2000). 'Stress' would involve all theories discussed above, including low temperatures, small size and larval mortality.

8. Conclusions

Particularly because of their comparatively simple body plan, ophiuroids are often considered as a basic taxon without special characters. In fact, a closer look is required in order to become aware of the variety and specializations of brittle stars. As discussed, the diverse class of Ophiuroidea displays manifold modes of reproduction, ranging from various asexual types and pelagic to non-pelagic development, including the extreme case of brooding large embryos in specialized brood chambers.

Gordon Hendler has carefully reviewed the literature on reproduction before the 1990s, and the patterns of that time hold true today. Hendler´s work probably covers all studies up until its time, including grey literature, and it is a complete and detailed compilation of all topics dealing with reproduction in ophiuroids. Since 1990, the studies of Maria Byrne and her colleagues have been a breakthrough in explaining the evolution of larval development. Simultaneously, they revealed the life history for a great number of species, most of them from the tropics.

Nevertheless, the reproduction and the mode of development remain unknown for many species, particularly in South Africa. In addition, a great amount of knowledge is based on observations that were made over a hundred years ago. Therefore, our idea of the South African brittle star fauna depends largely on dissections of specimens collected during expeditions of the 19th or early 20th century. Undoubtedly, Theodore Mortensen increased the numbers of species for which information about reproduction was available dramatically. However, his time spent in the region was limited and in the end he often dissected only a few specimens, making many of his findings somewhat

speculative. The overlooked case of brooding in the large and prominent coastal brittle star *O. wahlbergii* is a good example of why there might be many more undiscovered and exciting surprises. On a species level, there is a lot to learn about life history traits in South African brittle stars, which is also true for other regions of the world (Gillespie and McClintock 2007).

CHAPTER 2.

REPRODUCTION IN THE BROODING BRITTLE STAR *OPHIODERMA WAHLBERGII* MÜLLER & TROSCHEL, 1842

1. Introduction

South Africa has a rich ophiuroid fauna, with around 200 described species (Clark 1923; Mortensen 1933a; Clark 1974; Clark and Courtman-Stock 1976; Stöhr et al. 2012). The special geography of the coastline promotes variety and endemism. Prominent upwelling processes of the Benguela Current dominate the cold West Coast and the strong, southerly flowing Agulhas Current brings tropical water from the Indian Ocean along the East Coast. These contrasting environmental conditions result in a great diversity of marine species and generate a marked warm temperate transition zone along the South Coast, which is characterised by a high rate of endemicity (Awad et al. 2002). Ophiuroidea are the class of echinoderms with the largest number of species and most echinoderms, including ophiuroids, are broadcast spawners (Hendler 1991). However, knowledge about the life history of South African species is scarce (Branch and Branch 1992). In this study, the reproduction biology of the serpent-skinned brittle star *Ophioderma wahlbergii* Müller and Troschel, 1842 is examined. This species lives gregariously on subtidal soft-bottom habitats along the cold South and West coasts of South Africa and extends to the coast of Namibia (Branch et al. 2010).

O. wahlbergii is a large and locally abundant ophiuroid, but, apart from distribution records, almost nothing is known about the biology of this prominent brittle star. In their popular book about the marine life of South Africa, Branch and Branch (1992) first reported on this species brooding its young. The authors provided two images of the act of birth and mentioned that *O. wahlbergii* "produces no less than seven embryos", which are "about 2 cm in diameter". However, no further information is available about its ecology or reproduction. Current knowledge suggests that there is one species of *Ophioderma* in Southern Africa, which is *O. wahlbergii*, although specimens from the Bay of Lüderitz (Namibia) look slightly different to those in the Cape. The Namibian specimens have oral plates that are covered with a few, coarse granules (Bartsch 1974; Clark and Courtman-Stock 1976), whereas the ones collected in the Cape have oral plates that are covered with finer granules and resemble the type

specimen of '*O. leonis*', which is now a synonym of *O. wahlbergii* (Stöhr et al. 2014a, Stöhr 2014 pers comm).

The wide distribution of brittle stars can be regarded as a result of successful adaptations to different marine environments (Hyman 1955). Thus, it is not surprising that their diversity has also given rise to a variety of strategies of reproduction. Reproductive traits of brittle stars have been extensively discussed in Chapter 1. The ancestral form of broadcast spawning includes the development of a free-swimming ophiopluteus larva, which feeds in the water column until it has gained sufficient energy to metamorphose (Hyman 1955). Morphogenesis can be divided into two possible developmental paths. Either a barrel-shaped larva develops as an intermediate larval stage, called a "vitellaria", or this barrel-shaped larva does not develop and the ophiopluteus changes directly into the adult form (Strathmann and Rumrill 1987; Selvakumaraswamy and Byrne 2004). In addition, there is a general evolutionary trend from the self-feeding larva to a larva with maternally-provisioned yolk reserves (almost always the vitellaria) to a directly developing embryo, which, retained and brooded inside the mother, is protected from environmental risks and may be supplied with maternal nutrients.

Known brooding species of South Africa, other than *O. wahlbergii,* are *Amphiura capensis* (Djakonov 1914, in German), *Amphipholis squamata*, *Ophiomyxa vivipara capensis*, *Cryptopelta aster*, *Ophiomitrella corynephora* and *O. hamata*; *Ophiomyxa tenuispina* might brood, but this is unverified (Mortensen 1933a; Mortensen 1936). Unfortunately, no reproductive study of any of these species exists. Moreover, the two brooding Southern Ocean ophiuroids *Ophionotus hexactis* and *Ophiacantha vivipara* are recorded for Marion Island (Mortensen 1936). Olbers and Samyn (2012) suggested that *Ophiocoma brevipes* was a brooder, but this is questionable, as it seems more likely that young congeners use the bursae as a habitat. Almost all of the existing information about brooding species of South Africa comes from Mortensen's observations on collections for taxonomic purpose. These were based both on few specimens, and date back to the first half of the 20th century. Given the fact of little research effort and a high number of species in the region, it is thus likely that the provided list of brooding species of South Africa is incomplete. In fact, for about 30 years the report in a local field guide of *O. wahlbergii* being a large brooding species has remained unnoticed among international ophiuroid experts.

Of the nine (two from Marion Island) species listed above, seven are hermaphrodites or protandric hermaphrodites and this follows a clear, yet unexplained, correlation between small size, brooding and hermaphroditism (Mortensen 1920; Hyman 1955; Byrne 1991; Hendler 1991). In contrast, all species of the genus *Ophioderma* are large and gonochoric, meaning that they have separate sexes (Hendler 1991; Borges et al. 2009). This study takes advantage of the unique opportunity to analyse the remarkable brooding reproduction of one such large species, *O. wahlbergii*. As there is little information about ophiuroid reproduction in South Africa, investigations of brooding species from the region are both necessary and intriguing. This is the first comprehensive reproduction study about any brooding brittle star in South Africa and examines sex differentiation, numbers and morphology of brooded young and seasonality of reproduction in the brooder *O. wahlbergii*.

2. Methods

2.1. Sampling and study site

Ophioderma wahlbergii were sampled from the SAS Pietermaritzburg wreck in the Miller's Point area of False Bay, on the east coast of the Cape Peninsula (Cape Town, Western Cape, South Africa, GPS position S34°13.303' E018°28.465') using self-contained underwater breathing apparatus (SCUBA). The wreck lies at 20 m depth and *O. wahlbergii* populate the sandy bottom habitat adjacent to the hard substrate of the wreck. A sample of 20 brittle stars were collected from the edges and crevices of the wreck's body each month from June 2013 – May 2014 (n = 240). A standardized sampling technique of always collecting 20 adjoining specimens was applied to minimize selection bias. Due to boat availability and severe environmental conditions, the November sample was taken from Windmill Beach, a nearby on-shore site (GPS position S34°12.046' E018°27.397'). This location was located 2.9 km away from the main sample site and its depth was 2-5 m. Most samples were directly fixed in 70% ethanol. Some additional animals were kept and studied in the aquarium.

2.2. Morphological examinations

The disc diameter (dd) is the distance from the distal edge of a radial shield to the edge of the opposite interradius. Arm lengths of complete arms and dd were measured to the nearest millimetre using ruler and a vernier calliper respectively. All specimens

were dissected under a Wild dissection microscope, mostly by opening the ventral interradii. When the bursae projected into the dorsal disc side over the arms, clipping the arms at the mouth angles facilitated examinations of the pouches. All individuals were sexed, however, bursae of both females and males were examined the same way. In several individuals of different sizes, the sex was verified under the light microscope, using smear samples for male gonads. For all specimens of the January sample, the number of gonads was counted. In order to dissect the gonads undamaged, the disc was opened from the dorsal side, instead of the interradial ventral side. In females, for each bursa the ovary with the largest eggs was measured in length. Additionally, the number of eggs and diameter of the largest oocyte of this ovary was determined under the dissection microscope. In males, three testes were measured for each bursa. The width and length was determined for one radial testis and two interradial testes per bursae, measuring up to 30 testes per male. Because there were only two males present in January, five additional males from the February sample were examined.

In brooding females, the bursae were emptied and the juveniles counted per female and per bursa, and each juvenile subsequently measured. The maximum length of the embryos was measured when a disc had not yet been formed. In later embryonic stages, the disc diameter was measured under the dissection microscope for all individuals. When the embryo's disc deviated from a round shape, the diameter was averaged from two along and perpendicular measurements. Furthermore, number of arm segments were counted for juveniles of the samples November – April (six months). In the same way, disc diameter, number of arm segments and the length of the arms were measured for some of the largest brooded juveniles.

2.3. Data analysis

Exploratory and quantitative data analyses were performed in the R statistical environment (R Core Team 2011) on the RStudio interface (RStudio 2013). Normality and homogeneity of variances of samples were examined by eyeballing the data and using Kolmogorov-Smirnov tests.

2.3.1. Gonads, sex ratio and egg size

The numbers of gonads of females and males were averaged per individual and the means calculated for both the radial and interradial position. The mean and maximum numbers of eggs and egg size were calculated. A non-parametric Mann-

Whitney U test was used to detect size differences of brooding, as opposed to non-brooding, individuals. Furthermore, it was tested whether the testis size differed between the interradial and radial position. Deviations from expected frequencies in the sex ratio were tested by calculating the chi-square goodness of fit.

2.3.2. Embryos per female and seasonality

A Generalised Linear Model (GLM) was fitted to detect potential seasonal trends in the number of brooded embryos per female. Prior to further analyses, the most suitable GLM family was determined. Poisson and negative binomial distributions were considered as two alternative error models suitable for modelling count data (Zuur et al. 2009). Initially, a Poisson model was fitted to the data to identify potential over-dispersion, which is evident in cases where the variance is considerably greater than the mean. Should no evidence for over-dispersion be found, the response is presumed to follow a Poisson distribution. However, if the test indicates over-dispersion, the data presumably follow a negative binomial distribution, which is more suitable for count data with high variance. The candidate predictor variables included the categorical variables 'Month' and disc diameter in mm 'dd'. The optimal combination of predictor variables was selected based on Akaike's Information Criterion (AIC), with a lower value indicating a better fit (Zuur et al. 2009). Further, the AIC was used to judge whether candidate models with untransformed or log-transformed 'dd' variables resulted in the better fit. An analysis of deviance was performed to determine the variation explained by each added variable. The significance of the reduction of deviance by each added variable was tested using sequential χ^2-tests with a significance level of $p < 0.05$. The aim of this analysis was to evaluate a possible seasonal pattern, which could account for varying size class effects among months. The null hypothesis (H_0), that month has no significant effect on the number of embryos per female, was tested.

2.3.3. Developmental stages of brooded embryos

Based on the information of number of arm segments and dd for all brooded embryos from November to April, a linear model was fitted to predict the arm segments of embryos in the remaining samples. Embryos that had not formed a disc yet were excluded from the model. After the model was fitted, the brooded young were divided into size classes and according to these, divided into five different developmental stages: Stage 1, 0 arm segments (AS); stage 2, 0 - 9 AS + terminal plate (t), stage 3: 10 - 19 AS + t,

stage 4: 20 - 29 AS + t and stage 5: 30 - 39 AS + t. The abundance of the developmental stages per female and per bursa, as well as the simultaneous occurrence of developmental stages with each other, were examined.

2.4. Imaging and photography

One focus of this study was to create meaningful images in order to report on the numbers, sizes, appearance, position and orientation of the gonads, eggs and brooded juveniles. Several imaging techniques were applied. A novel Micro X-ray computed tomography method used to create 3-dimensional images of the juveniles inside the mother is described separately in Chapter 3. Images of *in situ* eggs and embryos, images of male and female gonads, and images of single juveniles were taken under a Nikon Stereoscopic Zoom Microscope SMZ1500 and smear samples of male gonads under a Nikon Eclipse 50i Compound Microscope, both using a Nikon DS Camera Control Unit DS-U2 & DS-5M Camera head. For scanning electron microscopy (SEM), embryos were treated with diluted household bleach (NaOCl) for a few seconds to remove the epidermis, as recommended by Stöhr et al. (2013). Thereafter, preparations were made according to the process used by Byrne (1991), but instead of a critical-point dryer, a few drops of hexamethyldisilazane (HDMS) were used. The embryos were then mounted on stubs, coated with gold palladium alloy and viewed and photographed under a Nova NanoSEM.

3. Results

3.1. Body size and sex

The 240 individuals studied ranged from 12 - 34 mm dd and had a mean dd of $24.5(\pm 5.1)$ mm. The arm median ranged from 23 - 125 mm with a mean of $7.6(\pm 1.92)$ mm. With the exception of seven indeterminate individuals and 11 individuals showing variations of hermaphroditism, the remaining 222 individuals were identifiable as either male or female. The overall sex ratio of males and females was 1:1.29, which was not significantly different from unity. The hypothesis that the sex ratio was 1:1 could thus not be rejected (chi-square test, n = 222, df = 1, $\chi^2 = 3.532$, $p < 0.05$). Although there was no difference between male and female sizes on average, the size classes >27 mm comprised about twice as many females as males, but males grew to the same size as females (Figure 4).

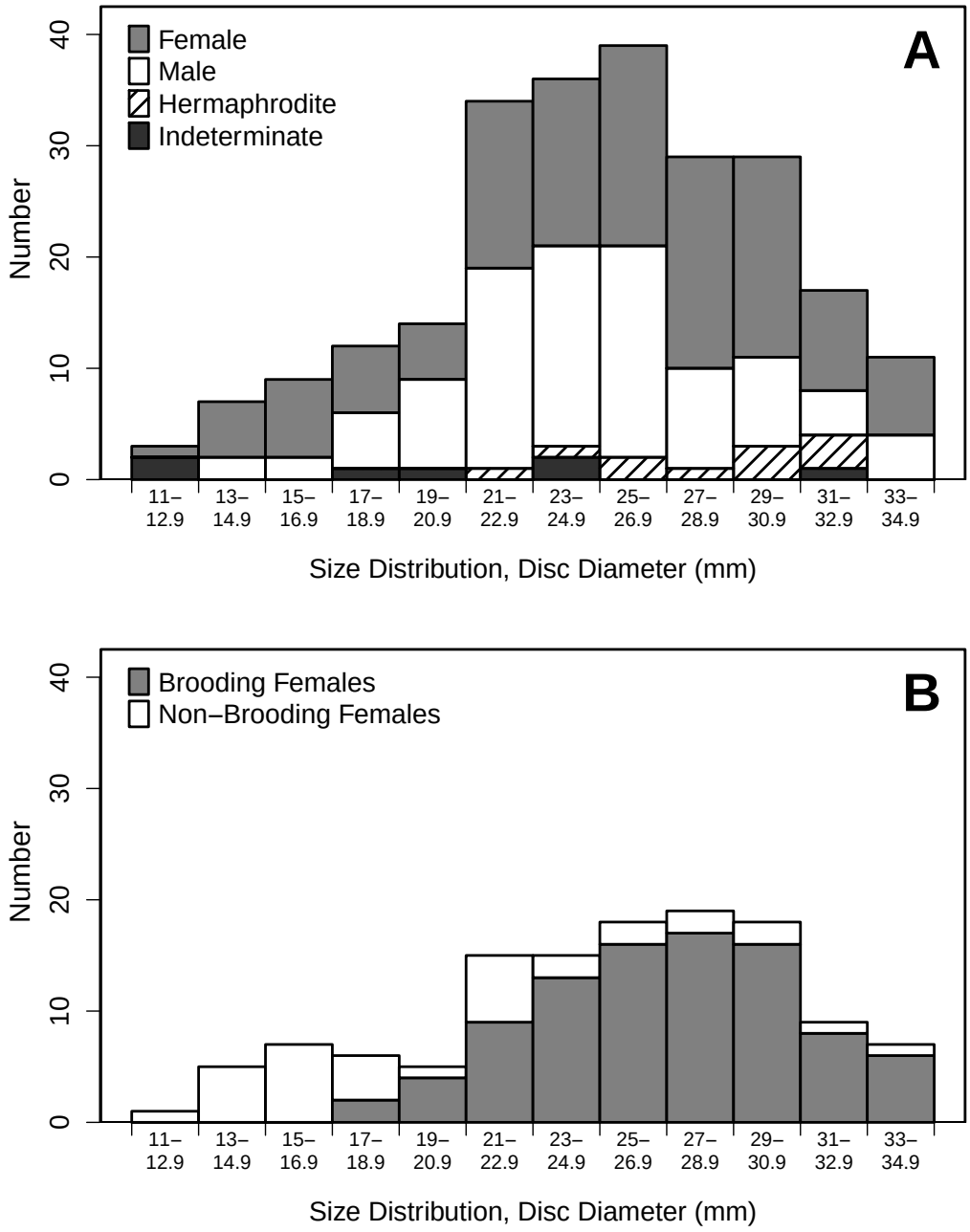

Figure 4. Size distributions of *Ophioderma wahlbergii*. **A** all individuals studied. **B** brooding and non-brooding females.

The vast majority of individuals had five arms but six individuals had only four arms (2.08%), three had six arms (1.25%) and one had seven arms (0.41%). Some individuals were found to have varying numbers of mouthpieces in comparison to numbers of arms, or arms were grown together at the base. Hermaphroditic individuals, which mostly fell into the larger size classes, had varying arrangements of male and female gonads. The majority were females with a few male gonads (female-dominated) and only three individuals with few ovaries and many testes were found (male-dominated). The latter did not brood any young. Most hermaphrodites had gonads of the

same gender connecting to the same bursa. However, two individuals were found to have both testes and ovaries connected to the same bursa. Ovotestes were not found, but no large numbers of gonads were viewed in histological samples. Five of the nine hermaphrodites brooded 1 - 2 embryos. Females reached maturity only after reaching 17 mm dd, resulting in brooding females being significantly larger than non-brooding females (Figure 4, Mann-Whitney-U test, $W = 2496.5$, $n_1 = 91$, $n_2 = 34$, $p < 0.001$).

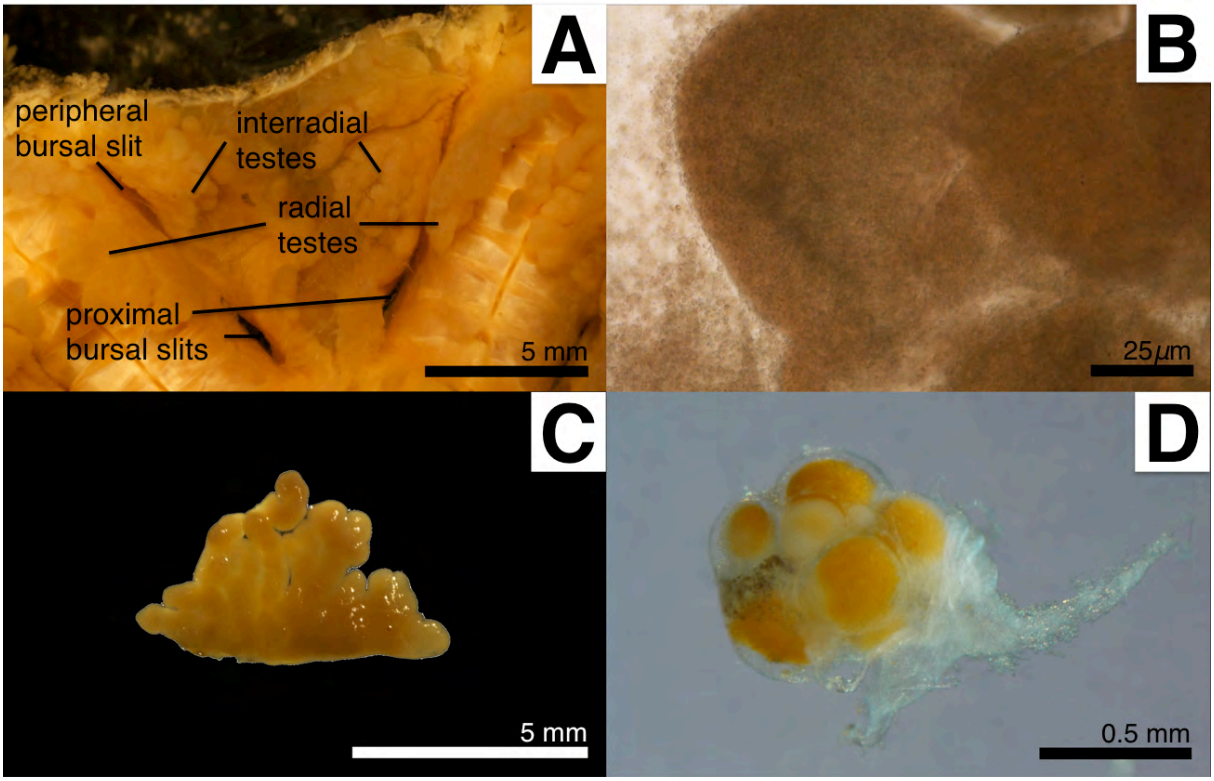

Figure 5. Gonads of *Ophioderma wahlbergii*. **A** *in situ* testes of 1 interradius (2 bursae), disc and stomach removed, bursae ruptured. **B** spermatozoa of smear preparation. **C** typical shape of a single testis removed from a bursa. **D** typical ovary with oocytes removed from a bursa.

3.2. Characteristics of the gonads, gametes and bursae

A clear gonadal, sexual dimorphism was found. The ovaries were small, spherical sacs averaging 0.707(±0.273) mm in length and females had a mean of 1.4(±0.7) ovaries on the radial side of the bursae, and 8.8(±2.8) ovaries interradially (n = 18). These findings resulted in a total mean average of 102(±32) gonads per female. The mean number of eggs in the ripest ovaries was 5.1(±2.8) and oocytes had a mean diameter of 0.25(±0.10) mm (n = 18). The largest oocyte measured 0.39 mm in diameter. Male individuals had a mean of 1.1(±0.3) testes on the radial side of the bursae, and 3.6(±0.7) testes interradially (n = 7). The testes were branched and significantly wider than long (Figure 5, C, width = 2.981(±0.584) mm, length =

3.379(±0.378) mm; Mann-Whitney-U test, W = 7, n_1= 7, n_2 = 7, p < 0.05). The size ((length+width)/2) of radial testes was significantly larger than of testes lying on the interradial side of the bursae (Mann-Whitney-U test, W = 35, n_1= 7, n_2 = 7, p < 0.05). Smear preparation of the testes revealed spermatozoa and verified the sex (Figure 5, B). Oocytes were found to be neutrally to positively buoyant in seawater.

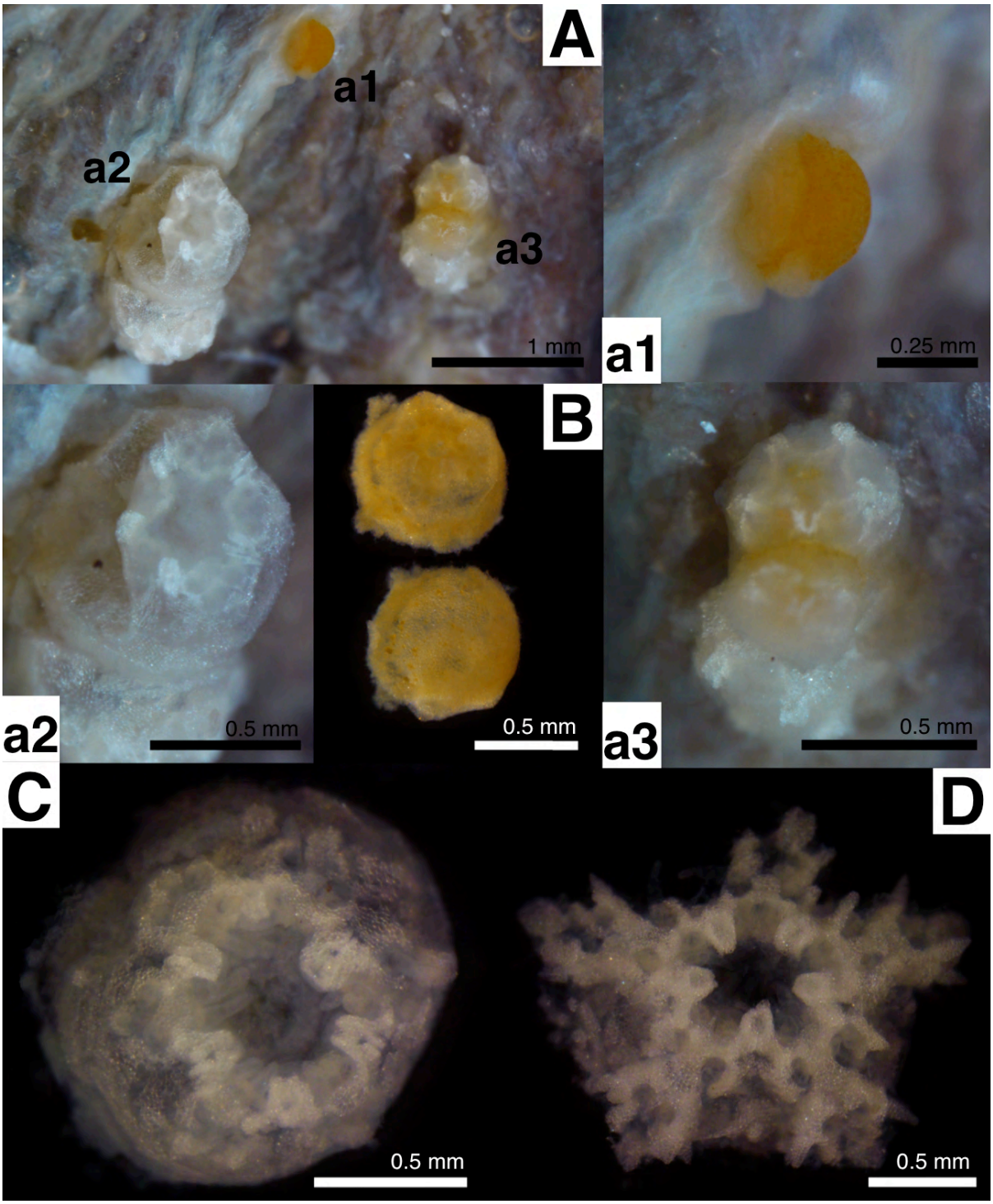

Figure 6. Early brooded young of *Ophioderma wahlbergii*, light microscopy. **A** *in situ* embryos in mothers bursa, attached to the bursal wall; **a1**, **a2**, **a3** enlarged. **B** oral (top) and dorsal (bottom) aspect of yolky embryo. **C**, **D** formation of terminal plates, arm segments, tube feet, mouth and disc.

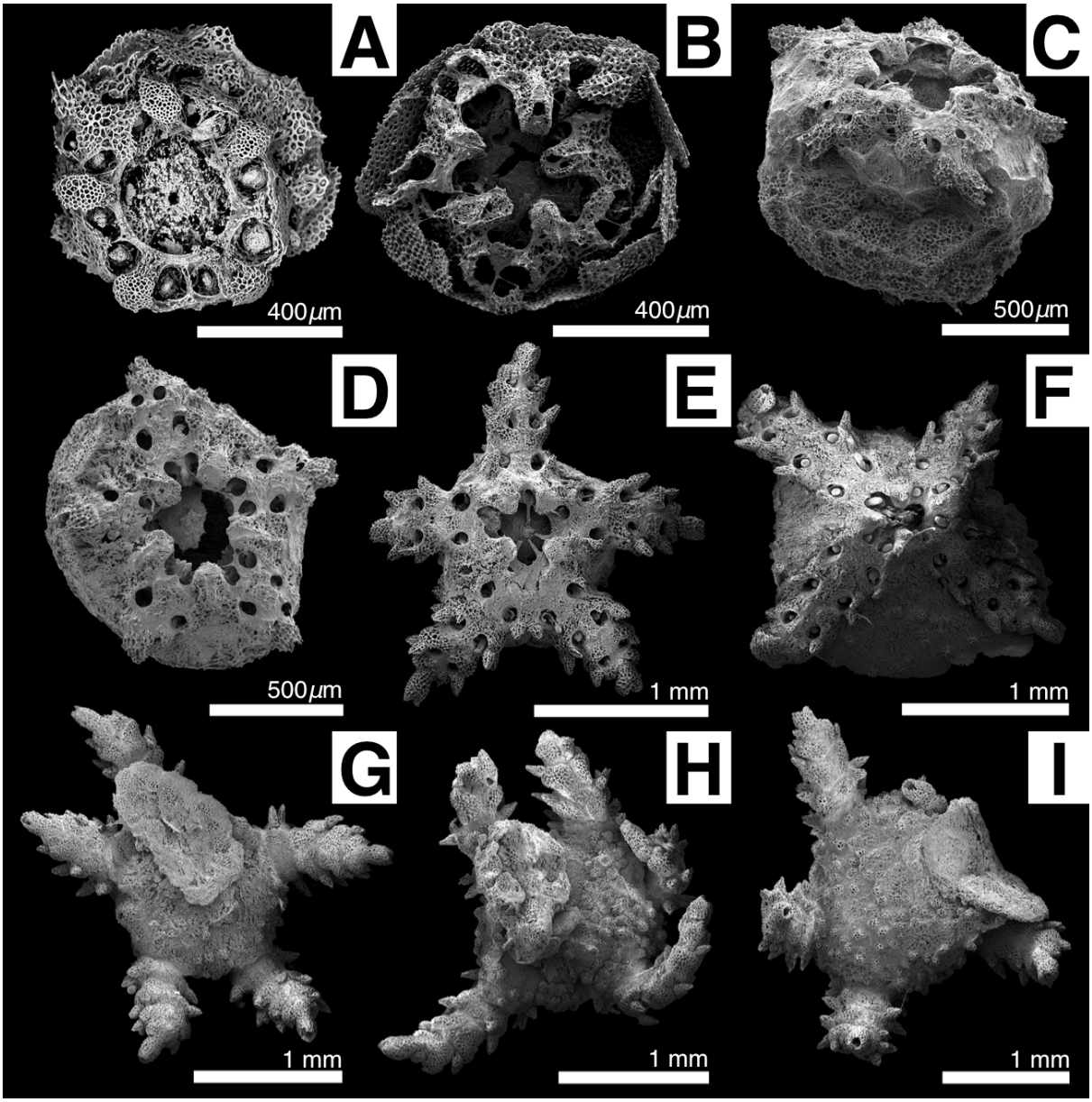

Figure 7. Earlier brooded young of *Ophioderma wahlbergii*, SEM images. **A-F** oral aspect of small size-classed juveniles. A mouth frame and terminal plates not clearly formed. **B** mouth frame visible. **C**, **D** forming arm segments and disc, enlarged calcified dorsal tissue. **E** arms, tube feet and mouth frame formed. **F** variation with 4 arms, note increased number of arm segments in comparison to E. **G-I** dorsal aspect of brooded juveniles, disc forming with developmental residue of calcified dorsal tissue.

3.3. Brooded young

A total of 640 brooded juveniles were retrieved from the samples. Of these, 183 embryos had not formed a disc and these had a mean size of 0.675(±0.200) mm. This group overlapped with the embryo class that had no terminal plate or arm segments. The 457 embryos that had at least formed a terminal plate had a mean dd of 0.298(±0.214) mm. The maximum dd was 9.33 mm, the maximum number of segments was 38 and the maximum arm length recorded was 18.67 mm, these maxima not being

from the same individual. There was a clear decline in the overall numbers of embryos from small to large size classes (Figure 9, B). Almost half of the embryos fell in the two smallest size classes from 0 to 4+t. The two largest size classes (30 - 39 + t) consisted of 18 juveniles (2.8%). Numbers of the middle size classes (10 - 24 +t) remained constant. Of the 457 young that had formed a disc and rudiment arms, two embryos had only three arms (0.45%), 16 had four arms (3.50%) and five had six arms (1.3%). These individuals were found in mothers with both four and five arms. One female brittle star with only four arms brooded 11 young, all with five arms.

Small individuals were loosely attached to the bursal wall by their dorsal side. The type of connection remained unknown, but there was a clear physical association with small embryos (Figure 6, A, a1-a3). This laminar juncture was easily breakable with a dissection needle or fine tweezers, and no channel or sinus was found that could connect to other organs supplying the embryo with nutrition. Instead, young embryos seemed to be nested into the tissue of the mother's bursa (Figure 6, a1). Almost all young embryos were surrounded by a yellow substance, which was assumed to contain yolk and lipids as an energy supply. Figure 6, C shows an embryo that lost its colour after it was retrieved from the bursa and kept in alcohol separately. Embryos that had not been preserved for long were generally orange-yellow (Figure 6, B). There were no signs of a physical connection between large young and the mother, but the stomachs of brooded young were always filled with an orange, yolky substance, which was highly positively buoyant in seawater and had the same orange colour as the ovaries.

3.3.1. Description and development

The dorsal disc, but not the arms, and then later, parts of the oral side and oral plates were covered with spherical, thorny granules (Figure 7 and 8, particularly a4). Juveniles of 2 mm dd had about 180 granules per mm^2 disc surface and the granules were irregularly spaced (Figure 8, a5). However, the stereom of granules, arm plates and spines was relatively regular (Figure 8, a1-a4). Moreover, the proximal arm plates were of triangular shape and rounded towards the lateral arm plates, which bore 3 - 5 short, conical spines (Figure 8, a1-3, a6). The distal-most segments, as well as younger juveniles bore fewer spines. The terminal plate had two thorns and was bulbous and hollow (Figure 7, I and 8, a2).

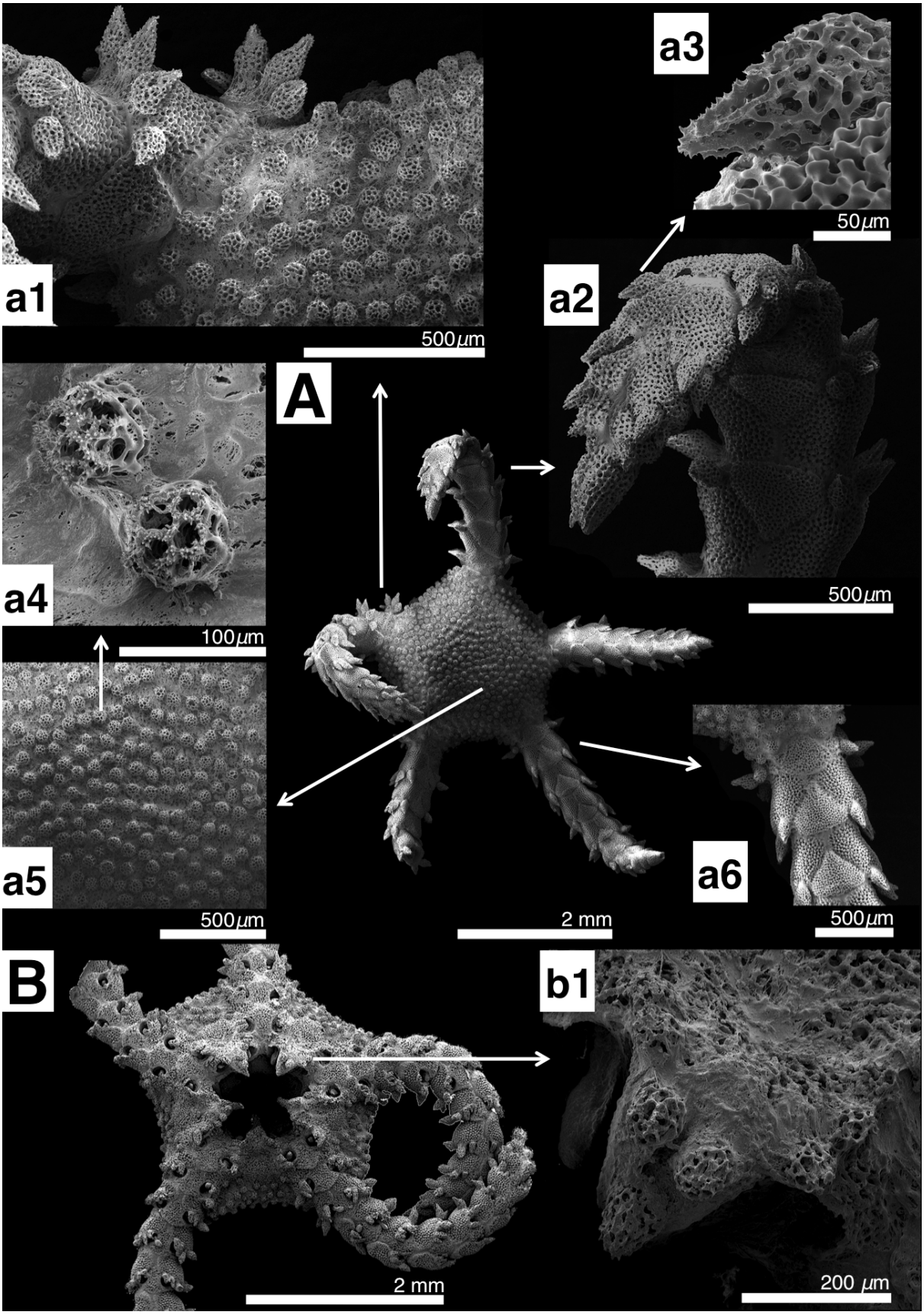

Figure 8. Later brooded young of *Ophioderma wahlbergii*, SEM images. **A** dorsal view of a medium size-classed embryo; **a1**, **a2**, **a4**, **a6** magnifications of A. **a3** arm spine; magnification of a2. **a5** disc granules, magnification of a4. **B** oral view of medium size-classed embryo; **b1** early jaw of mouth frame, magnification of B.

The mouthparts were the first structures to appear, indicating radial symmetry (Figure 6 and 7, A-B). According to the figures, the first tube feet formed, the terminal plate and the first arm segments could be seen, and also the mouth opened (Figure 6, C; 7, A). The formation of the disc was accompanied by the enhanced growth of dorsal, calcified tissue, still supported by the bursal wall. This tissue was then constricted to form the disc, leaving behind an adherent body on the back of the embryo, which was discarded at a later stage (Figure 7, G-I). Deformed "pieces of embryos" were found a number of times inside the bursae. These objects always had granules such as those on the disc. Furthermore, they were of different sizes and shapes. The deformed pieces were mostly larger than the original discarded residual tissue and seemed as if they had retained the ability to grow to a certain extent. After discarding the "calcified body of tissue", the young greatly resembled the adults, including arm spines and disc granules (Figure 8). However, the mouth apparatus of smaller-brooded young had not formed the strong jaws present in adults (Figure 8, b1).

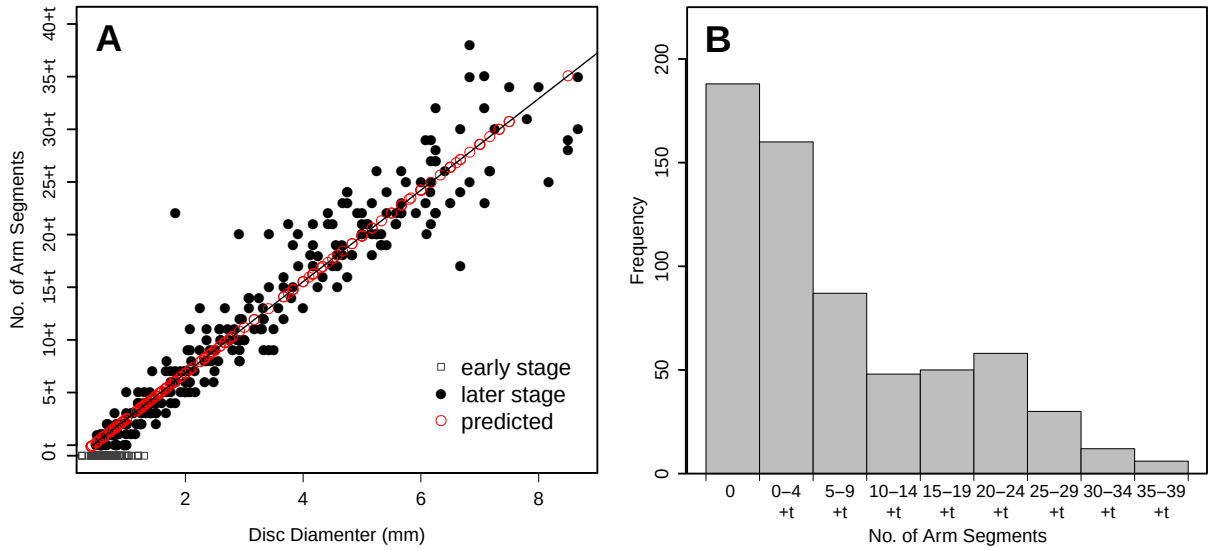

Figure 9. Sizes and number of arm segments of brooded *Ophioderma wahlbergii*. **A** Plot of number of arm segments against disc diameter (dd); *t* terminal plate, **B** histogram of size classes.

3.3.2. Juvenile release

The act of birth was observed in the aquarium and took about 15 minutes, and was also observed during a shore-dive at Windmill Beach. Even more interesting was a juvenile that successfully, and quickly, climbed back into its bursa after it had completely emerged, the only connection to the mother being a single arm holding onto

the bursal slit. In the laboratory and probably due to stress conditions, small embryos of about 1 mm dd were released and survived for several weeks. In addition, 18 larger juveniles of 2.5 - 7 mm dd were born, mostly during the first nights in the aquarium, but after two months in captivity. Of these 14 were ≥5 mm dd. They survived for a few months up to half a year, but none of them grew significantly. While adult individuals were easy to feed with larger pieces of fish or flakes, growth experiments with released juveniles (1 - 7 mm dd) failed.

3.4. Brooding behaviour

In total, 91 of the 125 females examined were brooding (72.8%). Among the monthly samples, the percentage ranged from 50% in July to 90% in March and the number of females per sample varied from eight in the July sample to 18 in January (n_{Month} = 20, Figure 10). Within brooding females, the mean number of brooded young was 6.9($\pm$5.9) embryos and the maximum number recorded was 33. The 11 hermaphrodite individuals were not treated as females.

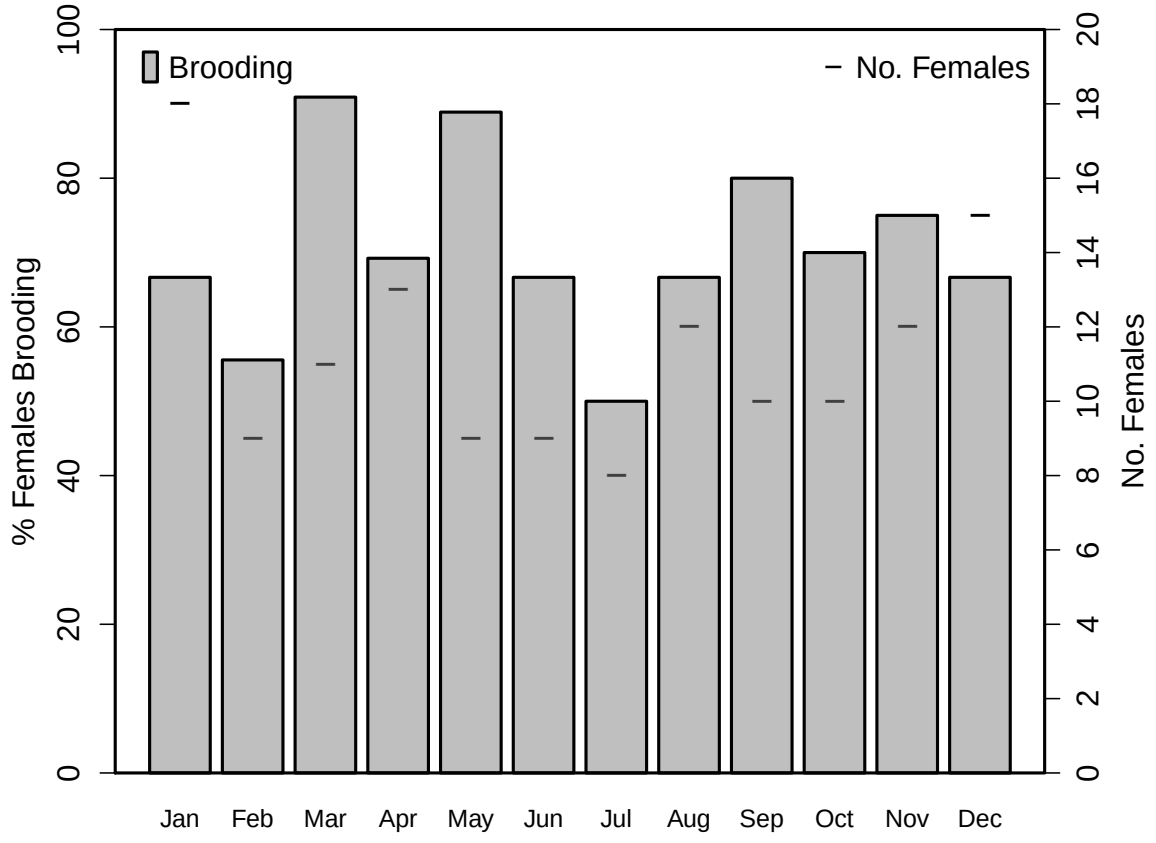

Figure 10. Percentage of brooding females and number of females per monthly sample.

3.4.1. GLM - Seasonality and number of brooded embryos

The dispersion parameter of the Poisson GLM was estimated at 5.021 (>>1), providing strong evidence for over-dispersion. The Negative Binomial GLM was therefore chosen for all further analyses, which was also supported by graphical inspection of residuals (Figure 11). The AIC was smaller for the candidate predictor variable 'log(dd)' than for 'dd'. Consequently, the model was fitted using 'log(dd)' and 'Month'. Adding 'log(dd)' to the Null model resulted in a lower AIC value and reduced the deviance by explaining 21.2% of the variation. Adding 'Month' further reduced the deviance by explaining 9.5% of the variation, but increased the AIC. Deviance reduction was significant for 'log(dd)' ($p < 0.001$), but not significant for 'Month' ($p < 0.0689$). Therefore, the most parsimonious model was found to contain 'log(dd)' as the only predictor variable of number of embryos (Table 4). The H_0 could not be rejected and it was assumed that, on average, there is no difference in brooded embryos dependent on months. Nonetheless, model predictions for the number of embryos may indicate a weak, but inconsistent seasonal pattern with highest numbers in the summer months February and December (11.9 and 8.3 embryos per female, respectively) and lowest numbers in the winter month June (1.6 per female, Figure 12).

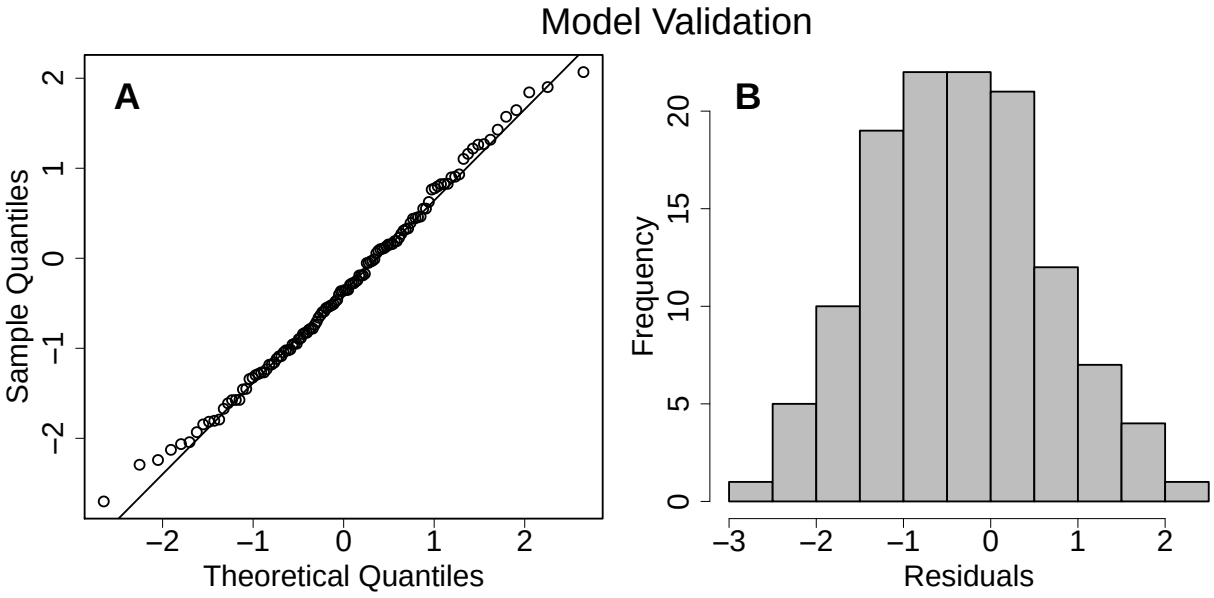

Figure 11. Model validation of the full model. **A** quantile-quantile plot **B** histogram of residuals.

Table 4. Deviance table of the Negative Biomial GLM analysis.

	Resid.	Resid. Df	AIC	Deviance	Δ Deviance	Variation explained	$p(\chi)$
NULL	123		668.9	200.01			
+log(dd)	122	1	638.4	157.16	42.847	21.2%	<0.001
+Month	111	11	643.3	138.58	18.588	9.5%	0.0689

Because the month effect was not significant, the expected number of embryos per female could be predicted as a function of 'log(dd)' for the size range of brooding females (17 - 35 mm). Based on the coefficients of the best-fitting Negative Binomial GLM, the relationship is given by the formula:

$$No.of\ Embryos = e^{-9.3227+3.3755*dd\ (mm)}$$

Females that just attained maturity (17 - 25 mm dd) were predicted to brood two to five embryos and the largest females (30 - 35 mm dd) may brood 15 embryos on average, but with higher variations (Figure 13).

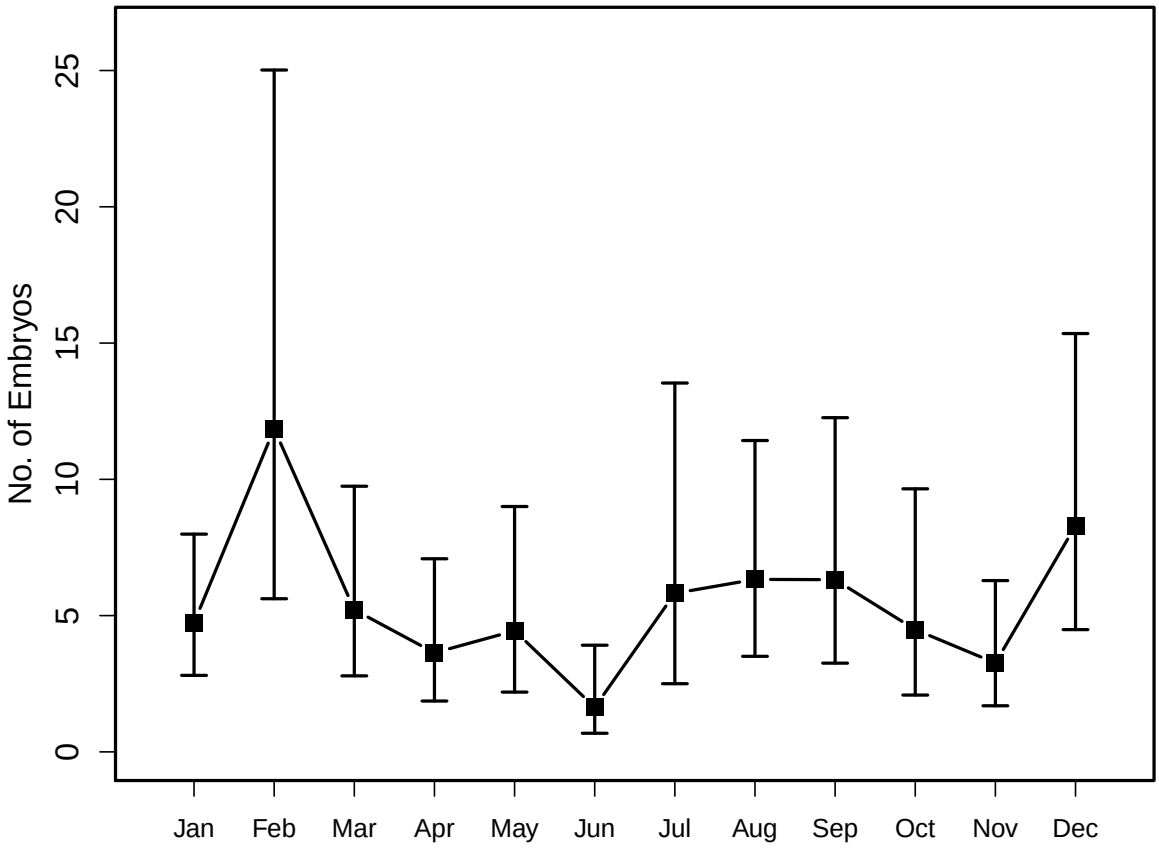

Figure 12. All-year brooding of *Ophioderma wahlbergii*. Plot of mean number of embryos per female as a response variable to month, when the disc diameter (dd) of females is kept constant at the mean dd of 26 mm (error bars show 95% confidence intervals).

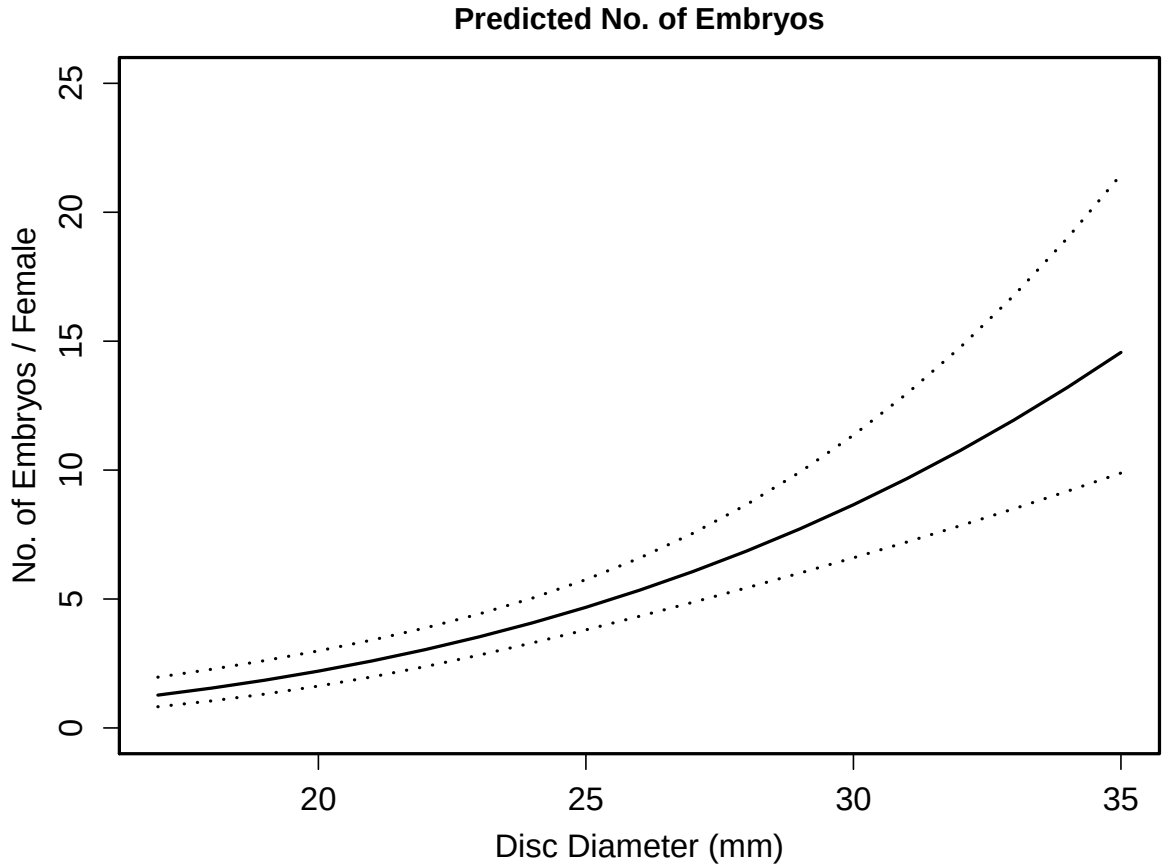

Figure 13. Embryo abundance as a response variable to disc diameter (dd) in mm for the range of mature female individuals of *Ophioderma wahlbergii* (dotted lines indicate 95% confidence intervals, *formula:* $NoEmbryos = e^{-9.3227+3.3755*dd\ (mm)}$).

3.4.2. Stages within individual brooding females

Individual females brooded a variety of developmental stages and 34% accommodated two different stages; 13% contained four and at least 2% contained all five of the distinguished stages (Figure 14, A). In contrast, 85% of the individual bursae contained only a single stage; 0.5% brooded three stages and no bursa had more than three (Figure 14, B). Figure 14 (C, D) further depicts how the combinations of the distinguished developmental stages varied between individual females and individual bursae. In both individual females and individual bursae, numbers were highest for co-occurrences of identical stages. While co-occurrence of small stages with large stages (1, 2 with 4, 5) was found in whole females, finding small stages in the same bursae together with large stages was rare. For example, there was no stage 5 occurring together with a stage 1, stage 2 or stage 3. Moreover, the stages 3 and 4 were only found once with a stage 1. The co-occurrence of a stage 2 with stage 4 was reported six times.

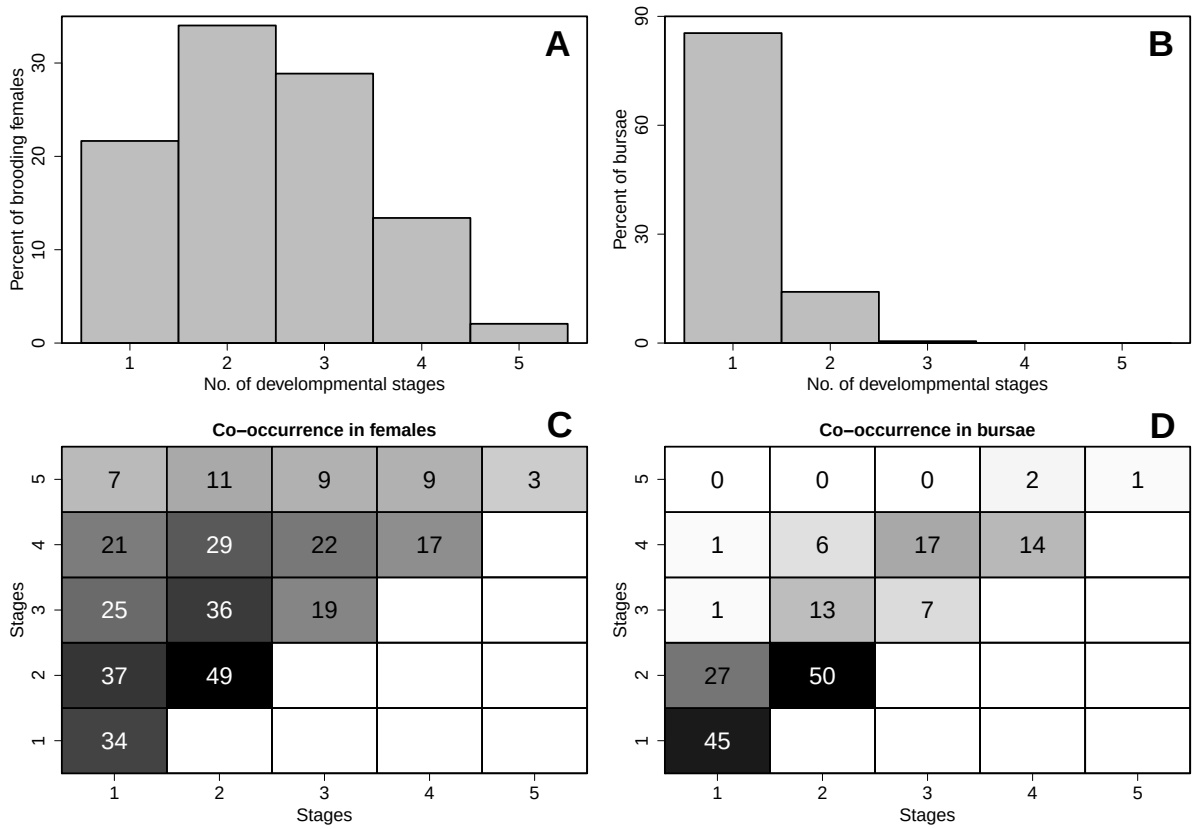

Figure 14. Number and combination of developmental stages brooded in *Ophioderma wahlbergii*. Co-occurrence matrices show the number of times a stage was found with another stage. Combination of one stage with more than one stage was still counted as one. The same principle was applied to identical stages, i.e. two 1's and two 2's were counted 1x1=1, 1x2=1, 2x2=1. **A** number of stages in individual brooding females (n=91). **B** number of stages per bursae (n= 390). **C** number of co-occurring developmental stages in individual brooding females. **D** number of co-occurring developmental stages in bursae of brooding females.

4. Discussion

4.1. Body size and sexuality

The reproduction type in *Ophioderma wahlbergii* is interesting, particularly due to its large body size and the separated sexes. Generally, brooding ophiuroids have a small disc diameter of 10 mm or less, but this species is large (>30 mm dd) and actually resembles a typical broadcast spawner (Hendler 1975). It may be possible that the case of brooding in *O. wahlbergii* remained unnoticed for such a long time, because Mortensen and other earlier echinoderm experts looking for brooding brittle stars focussed on small species.

Moreover, the equal number of separate female and male individuals and the additional histological examinations show that *O. wahlbergii* is gonochoric. Therefore, it

also violates the second trend of the majority of brooding species being hermaphrodites or protandric hermaphrodites (Hyman 1955; Hendler 1991). Hermaphroditism, including self-fertilization, seems to be advantageous in widely dispersed or sluggish organisms, when reproductive contacts take place infrequently (Heath 1977). These characteristics are met for most small ophiuroids, but are not characteristics for the mobile *O. wahlbergii*. Anecdotal reports of aggregations and comparatively fast migrations exist and could therefore explain why gonochorism is preserved in this large brittle star. Nevertheless, the 5% of individuals showing hermaphroditism are a high percentage of sexual abnormalities. Hermaphrodite individuals can be found among gonochoric ophiuroids, but normally occur in <1% of the population (Hendler 1991; Hendler and Tran 2001). Hendler and Tyler (1986) and Borges et al. (2009) respectively found a single hermaphrodite among 100 sampled *O. brevispinum* and *O. januarii*, both broadcast spawners. Therefore, considering the background that hermaphroditism is correlated with brooding species, the noticable higher numbers of hermaphroditic individuals compared to closely-related species might not just be a meaningless natural deviation, occurring by chance. However, the true nature of the 5% of hermaphroditic incidents remains unknown. At least, the observation of hermaphrodites brooding embryos shows that these individuals are fertile. On the other hand, none of the female-dominated hermaphrodites brooded a substantial number of young and it is unclear to what extent their male gametes fertilize own oocytes or the eggs of other individuals. Also, because none of the male-dominated hermaphrodites were found to brood, the fecundity of the female side of the hermaphrodites is possibly lower than of pure females. In any case, the high percentage of hermaphroditism does not negate the general gonochoric character of *O. wahlbergii*.

Although numbers of large males were lower than of large females, a complete sex-change, such as protandry, seems unlikely. There is no evidence for a simultaneous decrease of males associated with the increase of large females. Further, the gonads are very different in shape and size and there are no intra-gonadal signs of a sex change. However, the hermaphrodites fell into the larger size classes, suggesting that these individuals grow gonads of the opposite sex only at a larger stage. Hence, a small proportion of 5% of the population partially changes sexes.

It is difficult to explain the low numbers of males in the larger size classes, but it might be possible that large males have larger home ranges and undergo migrations. In the months December and January, males, particularly large ones, were almost absent

from the samples (Figure 10). The study site was an isolated shipwreck surrounded by sand and gravel soft-bottom habitat. It is possible that half of the males with >27 mm dd leave the wreck and undergo migrations in search of other populations. Thereby, they could maintain sexual connectivity and the advantages of gonochorism, but this hypothesis demands verification.

4.2. Gonads and oocytes

Sexual dimorphism with regard to number and gonad shape is not uncommon among brittle stars, but size, number and shape of the gonads are generally not meaningful for ecological or phylogenetic conclusions (Hendler 1991). Yet, the intraspecific gonaldal size difference between male and female *O. wahlbergii* is striking. In males, the testes can take up a large proportion of the coelom, which is needed for the brooded young in females. While the large testes indicate a high number of sperm, an average of five vitellogenetic oocytes in the small sac-like ovaries results in about 500 oocytes per individual. This number is 3 - 4 times less than in other species of *Ophioderma* and the late-vitellogenetic oocyte diameter of $0.25(\pm 0.10)$ mm is comparable to related species with abbreviated development and a lecithotrophic vitellaria larva (references in Hendler 1991). The number is still high considering the small number of embryos, but the large variation in diameter indicates that not all eggs ripen at the same time. Oocytes of the broadcast spawning population of *Ophioderma longicauda* take more than a year to mature (Fenaux 1972, cited as in Hendler 1991). Borges et al. (2009) measured a very large oocyte of 0.489 mm for *Ophioderma januarii* with a presumably (but unverified) lecithotrophic reproduction. Hendler and Tyler (1986) reported on a maximum oocyte size of 0.35 mm in *Ophioderma brevispinum*. Therefore, the maximum oocyte diameter of 0.39 mm in this study is the second largest measured for the genus.

4.3. Brooded young

In the past, there were misinterpretations regarding brittle stars brooding young, because the individuals found inside bursae do not necessarily have to belong to the "mother", and not even to the same species (Mortensen 1920; Hendler et al. 1999; Morgan and Jangoux 2004). Although this study lacks confirmation using genetic tests, finding young embryos only in females and in various developmental stages indicates that the small ones are descended from their mothers. In addition, emerging young

largely resemble the adult appearance. The morphological characteristics, such as the granulated discs of both adults and young, are in strong agreement with this theory. After all, *O. wahlbergii* is the only species of the genus known to the region and cannot be confused. All these arguments are in favour of *O. wahlbergii* being a brooder.

4.3.1. Size

The size of the largest brooded young with almost 9.3 mm dd is exceptional for bursal brooding. Among all known brooding ophiuroids there is only one that rears embryos of a similar size, which is *Ophionotus hexactis*, an intraovarian brooder that has young with a maximum of 8.3 mm dd (Mortensen 1921; Morison 1979, as cited in Hendler 1991). A general rule for brooding ophiuroids suggests that a larger propagule size is more advantageous for the survival of juveniles (Hendler 1975). Undoubtedly, the juveniles' size is limited to the body size of the parent and, as *O. wahlbergii* is one of the few large brooding species, it is capable to brood large young. Relative to the body size, small species may brood even larger embryos (see Chapter 3).

4.3.2. Development

Given the fact that no clear features of a pelagic larva were observed during the extraction of 640 embryos, it can be concluded that *O. wahlbergii* is a direct developer. The enhanced growth of the embryonic dorsal tissue is unknown for ophiuroids. The fact that it was observed in numerous individual embryos shows that it is a general developmental characteristic and not just an abnormal growth pattern (Figure 7, C-D, G-I). The SEM images revealed that the enlarged tissue contained calcified plates and that its constriction forms the disc, leaving a small residue on the dorsal side of the disc. The function of this "calcified dorsal body" is unknown, but at first glance it seems like a waste of resources. It could, however, serve the young embryo as an increased surface to take up nutrients, but this is speculative. The fact that the body is still large in embryos that have already formed a mouth is an aspect in disfavour of this theory. The deformed living "pieces of embryo" in the bursae are thought to originate from the discarded calcified dorsal bodies. This would show that this tissue is vital and able to undertake physiological functions to support the growing embryo. Another theory is that the appendage is needed as connective tissue to the bursal wall.

The percentage of embryos with varying numbers of arms was very similar to the percentage in the overall adult samples. This indicates that the abundance of abnormal

arm numbers is determined at a developmental stage. Individuals with four or more than five arms do not have any noticeable disadvantages, but embryos with three arms do not survive to reach maturity.

4.3.3. Association with the mother

The finding that embryos have a physical attachment to the bursal wall is exciting and it highlights the advanced brooding adaptation of this species. When shed into the bursae, the positive buoyancy of the eggs probably facilitates the nesting of the small embryo and reduces direct losses through the bursal slits, which open towards the bottom. To date, a true connection between the adult and the young has only been reported for *Amphipholis squamata*, a small-sized species complex found globally. Although *A. squamata* is one of the few verified viviparous ophiuroids, the connection anchors the embryo, but does not serve any nutrient-provisioning function (Fell 1946). Because the embryonic attachment in *O. wahlbergii* is superficial, easily breakable and disappears at later stages, it seems to serve a similar function.

4.3.4. Nutrition

The orange colour of the eggs and of early embryos suggests that, until it becomes deficient, yolk is the first energy supply. The further supply of nutrients is speculative, but considering the large size, it is probable that *O. wahlbergii* is truly viviparous too. Embryos of *A. squamata* take up nutrients directly from the seawater, from endosymbiontic bacteria and very likely from the bursal wall (Walker and Lesser 1989). Most notably the latter is also applicable for *O. wahlbergii*, because larger juveniles press their mouth against the bursal wall (see Chapter 3). In addition, the gut content of embryos, containing a yolky substance of the same colour as the oocytes, implies the consumption of nurse eggs. Adelophagy, the ingestion of siblings, is not ruled out and would further explain the decreasing numbers of young, as they grow larger. However, in combination with the production of relatively few eggs, both strategies are proven to provide insufficient energy for the growth of such large young and instead, the main energy source must come from an external source, or parental body fluid (Turner and Dearborn 1979). Larger young can stick their arms and even body through the bursal slits. By doing so, brooded young of *O. wahlbergii* may catch small food particles, but this does not explain the growth of smaller stages, which have less-developed arms. Without measures of control the bursal walls of pregnant females were found to be a

stronger organ than in non-pregnant individuals, or males, and they were both more flexible and subjectively thicker. Towards the inside of the bursae, the walls were further wrinkled and often covered with a yellow substance of unknown content and origin. Because nurse eggs, adelophagy and external food sources may be a source of nourishment, but are no sufficient energy supply, the bursal wall is arguably the main nutritional source. Although it could not be proven in this study, structures of the bursal wall probably hold the answer to the true trophic interaction of adults with the embryos.

4.3.5. Fate of the embryo

The gradual decrease in numbers of brooded young towards the larger size classes could be explained by rates of mortality, or the young propagules being released at different sizes (Figure 9, B). So far, it was unkown whether young brittle stars are born at varying stages and sizes (Hendler 1975). The young of the asteroid *Patiriella vivipara*, an intraovarian brooding species, leave the mother at a wide range of sizes of 1.4-5.0 mm diameter (Byrne 1996; Prestedge 1998). In the aquarium, the majority of *O. wahlbergii* gave birth soon after the pregnant females were collected. This shows that the mothers can release viable propagules at a range of sizes. With the information of mostly larger juveniles born after being captured, the hypothesis is that normally juveniles leave the adult at >5 mm dd. If the conditions are unfavourable or change, they can release even the smallest embryos. However, when environmental conditions, such as food availability, are good, young are probably retained until they reach their maximum size of 9.3 mm. The failed growth experiments show that juveniles have different habitat and food requirements than adults. Although it was looked for small free-living individuals among the adults, there were hardly any juveniles between 8 - 12 mm dd. Because juveniles in the aquarium did not bury themselves, they probably hide in smaller crevices and ground covering in the wild, where they are difficult to find.

4.3.6. Act of birth

Birthing takes several hours in *A. squamata* (Fell 1946), but in *O. wahlbergii* it takes only 10 - 20 minutes, similar to *O. hexactis* (Morison 1979, as cited in Hendler 1991). Turner and Dearborn (1979) argued that some embryos of *O. hexactis* might be unable to leave the ovaries and bursae due to their large size. In *O. wahlbergii*, the bursal slit widens without damage to the adult disc and the young crawl out using pulling

movements of the arms. The act of crawling back into the bursae reveals how expandable the bursal slits are and here, mutable collagenous tissue seems to play an important role (see Chapter 1, Section 6.4.2.).

4.4. Length of brooding

The time it takes from fertilization to the release of the juveniles is difficult to estimate from the results, since reproduction was continuous and hence growth rates could not be inferred from seasonal changes in embryo size-distribution. Young of *A. squamata* are brooded for 3 - 6 months (Hyman 1955; Hendler 1975; Rumrill and Pearse 1985), but the embryos are tiny compared to those of *O. wahlbergii*. The observation of surviving, but not significantly growing, young in the laboratory indicates that the time of brooding in this species could be similarly extended as in *O. hexactis*, where embryos undergo a period of 2 - 3 y of brooded development (Morison 1979, as cited in Hendler 1991). *O. hexactis* is a polar species with probably slower growth rates, but considering even larger embryos in *O. wahlbergii*, a similarly long developmental time would not be surprising.

4.5. Number of young

Most brooding ophiuroids brood a few to about 50 embryos (Hendler 1991). The small *A. squamata* can accommodate up to 25 embryos, the medium sized *Amphiura capensis* up to five and the large *O. hexactis* up to 54 embryos (Djakonov 1914; Mortensen 1920; Hendler 1975; Morison 1979, as cited in Hendler 1991). With an average of seven and a maximum of 33 brooded young *O. wahlbergii* fits well into this range. Nevertheless, the maximum number arose from many young in early stages, so the maximum number of large young is around 15 (also see Chapter 3). However, the more interesting question is how many juveniles can be produced within a year? Unfortunately, because the data reveal no seasonal signal, the answer can only be speculated on. If one would assume that viable juveniles leave the adult at >5 mm dd, which is equivalent to >20 arm segments, then in this study and for the duration of one year, 106 young were large enough to leave the mother. Divided by 91 brooding females, a female of *O. wahlbergii* releases only an average of 1.16 juveniles a year. At first, this number seems to be very low, but considering a long lifespan of this large brittle star, the number is plausible. Other species in the genus *Ophioderma* may reach the age of 30, which is a conservative number (Hendler 1991, and referenced therein). The low

number of free-living juveniles would also explain why they were difficult to find during the dives. In comparison, Morison (1979, as cited in Hendler 1991) estimated that *O. hexactis* produces about eight juveniles per adult per year, but this species has a shorter lifespan of 20 y and the largest individuals lose their fecundity.

4.6. Seasonality of brooding

It has been suggested that brooding species have an extended spawning period compared to broadcast spawners, but in fact, this relationship is unknown (Hyman 1955; Hendler and Tran 2001). Species with a recent evolution of brooding like the brooding population of *O. longicauda* still have a very pronounced brooding seasonality (Stöhr et al. 2009). Nevertheless, the brooding season of many ophiuroids can extend over months (Hendler 1991). This is also the case for *O. wahlbergii,* where the consistently high percentages of pregnant females show that this species broods all year round. Finding young embryos, as well as males with large testes, at all times further suggests that there is constant gamete release throughout the year. If reproduction were seasonal, the number of embryos per female would be expected to change, but the statistical tests did not detect any significant difference in the average number of embryos among months. They are, therefore, in agreement with the theory that brooding of *O. wahlbergii* does not follow any seasonal trends. In climate conditions with no pronounced seasonality, this is not necessarily surprising. In fact, marine invertebrate species of the South African West Coast do not follow any consistent pattern of reproductive seasonality, but many breed all year round (Hodgson 2010). However, in this study the low sample size did decrease the power of the test and there were some variations of brooded young between the months, so additional sampling may reveal weak seasonal trends. In contrast, the relationship of a larger female size resulting in more brooded juveniles was clearly recognisable.

Hendler (1991) differentiated between sequential and simultaneous brooders. The former are species brooding only one cohort of embryos and the latter continuously release offspring of different size. Although *O. wahlbergii* belongs to the group of species brooding different sizes of embryos at the same time, the individual bursae contain sequential clutches (Figure 14). Therefore, like the deep sea species *Amphiura carchara*, *O. wahlbergii* shows characteristics of both groups (Hendler and Tran 2001). The sequential brooding reveals that all gonads of a single bursa are synchronized. Nevertheless, the constantly high numbers of brooding individuals may mask females'

recovery phases. Because it is not possible to track the reproduction of a single individual over a long period, females may discontinue brooding after a long brooding phase. This would serve as one explanation why there are always females that are not brooding any young.

4.7. Evolution of brooding in *Ophioderma*

Brooding in *Ophioderma* is a recent discovery, but several other brooding ophiodermatids are known (Byrne et al. 2008). With their lecithotrophic larvae, they are suggested to be able to shift towards a brooding behaviour (Byrne 1991; Cisternas and Byrne 2005). The key step towards brooding evolves via an ovoviviparous behaviour of retaining self-sufficient eggs inside the bursae. Species brooding a pelagic-type larva provide a link to such first brooding forms (Byrne et al. 2008). However, without additional food supply, the growth of these embryos is limited and determined by the amount of own yolk reserves. Brooding in *Ophioderma* was reported for the comparatively well-known Mediterranean species *O. longicauda*, which is a species complex with a brooding subpopulation (Stöhr et al. 2009). Here, the adaptation is thought to be evolutionarly young, probably only about 1-2 million years old and has evolved as a strategy to bypass hyper-oligotrophic, pelagic conditions (Boissin et al. 2011). In contrast, in *O. wahlbergii* the brooding of enormous young, the lack of larval structures and the unverified but likely nourishment of the young all support a much older reproduction strategy. Furthermore, the cause of the brooding adaptation is entirely speculative, but factors like small size, strong seasonality or oligotrophic conditions are not met. In fact, the coastal upwelling on the West Coast of southern Africa supplies plenty of nutrition, but the strong currents might be dangerous to pelagic larvae. However, it is possible that the conditions varied greatly when the first individuals of *O. wahlbergii* developed the strategy of brooding.

CHAPTER 3.

HOW MANY INSIDE? A 3D μCT SCANNING TECHNIQUE FOR VISUALIZING INTERNAL STRUCTURES IN BROODING OPHIUROIDS

1. Introduction

Soon after Röntgen's discovery that X-rays can uncover internal bone structures, the first application of X-rays, dating back to more than a hundred years, was a medical procedure (Spiegel 1995). Almost 80 years later, Computed Tomography (CT) was developed by means of acquiring object projections in different directions and computing them into virtual slice stacks, which are then used to reconstruct a 3-dimensional (3D) model (Hounsfield 1973). Since then, this method has mainly been used in medical applications investigating large-scale biological bodies. Over the years, resolutions at submicrometer scale have become achievable, extending the application possibilities to smaller samples (Baruchel et al. 2008). Among other micro 3D imaging techniques, there are wide 3D applications for morphological studies in marine biology, but so far, they have scarcely been used (Boistel et al. 2011). In echinoderms, µCT and Magnetic Resonance Imaging (MRI) have been applied, which are both non-invasive tomographic imaging techniques using 3D scanning protocols (Ziegler 2012). While MRI is used to visualise internal organisation of soft tissue, µCT studies are suited to detect the calcified structures of echinoderms (Ziegler et al. 2008; Ziegler et al. 2011). Studies applying MRI techniques are more frequently used than approaches using µCT, although the latter method can achieve considerable results for soft-bodied marine invertebrates too (Dinley et al. 2010). In this study, µCT scanning based on the attenuation contrast between different materials, is applied to brooding brittle stars, which are small to medium-scale biological samples from the marine environment and include soft tissue and hard, calcified structure.

Brooding is an advanced reproduction strategy in ophiuroids, commonly known as brittle stars. Instead of a pelagic larval period, the young are retained inside universal sacs called bursae, and raised to a crawl-away stage. Until now, information on the numbers, size, orientation and posture of brooded embryos inside the bursae has been based on observations from disturbed and/or preserved samples (Hendler 1991). For centuries, dissection has been the only way to examine brooded embryos, and, to the author's knowledge, there is no descriptive *in situ* visualization where the surrounding

structures, like the bursal wall, are left intact. In Chapter 2, brooding of *Ophioderma wahlbergii* was described for the first time. This subtidal species from shallow-waters of South Africa has separate sexes and females brood enormous young of different cohorts. In fact, the young are among the largest to occur within the Ophiuroidea. Brooding in such a prominent (35 mm disc diameter (dd)) and gonochoric brittle star is a rather unusual circumstance. Most brooding species are small and hermaphrodites (Mortensen 1920; Hyman 1955; Hendler 1991). *Amphipholis squamata* (Delle Chiaje 1828) fulfils these characteristics, as it is a tiny, hermaphroditic species complex found worldwide, including in the intertidal zone of South Africa. Only the minority of ophiuroids are brooders and brooding with a direct development is considered as the highest evolved reproduction mode (Hendler 1975). Therefore, selection pressures have been intensely discussed and small body size has been proposed to facilitate the evolution of brooding (Menge 1975; Strathmann and Strathmann 1982; Poulin and Féral 1996; Gillespie and McClintock 2007). But how do the brooding strategies of a small, hermaphroditic ophiuroid differ compared to a large, gonochoric species? Here, two distantly-related brooding brittle stars with an appearing congruent reproduction strategy are compared by a novel 3D analysis. The aim of this study is to visualise the brooded young of both species *in situ* and to create the first 3D models of brooding brittle stars. For the first time, volume calculations of the devoted body space for the brooded embryos are presented and the brooding strategies of both the small and the large ophiuroid species are compared. The models are demonstrative, serve various teaching objectives and can also be printed in 3D.

2. Methods

2.1. Sampling and study site

Ophioderma wahlbergii were sampled from Windmills Beach on the False Bay coast of the Cape Peninsula (Cape Town, Western Cape, South Africa, GPS position S34°12.046' E018°27.397') and *Amphipholis squamata* were collected at Mouille Point (GPS position S33°53.932' E18°24.573'). Of both species, 20 large specimens likely to brood embryos were taken into the laboratory.

2.2. Inspections of brooding and scanning

In the laboratory, all individuals were examined for signs of brooding. Using fine

tweezers, the bursal slits of *O. wahlbergii* were carefully investigated for arms of brooded juveniles. All *A. squamata* were looked at under the dissection microscope using bottom light shining through the disc to detect brooded young. The five individuals of both species that were thought to carry most embryos were then fixed in alcohol and viewed in the preview of the µCT scanner. In the preview the number and size of the embryos were evaluated by eye and consequently the individual that seemed to devote the most space to brooding young was chosen. Without any further preparation, the final specimen of each species was mounted on Styrofoam (arms cut off) and scanned with a General Electric Phoenix V|Tome|X NF180 at $(30\ \mu m)^3$ and $(9\ \mu m)^3$ (*O. wahlbergii*) and $(3\ \mu m)^3$ voxel resolution (*A. squamata*).

2.3. 3D visualization

The µCT scanner was set to generate a 16bit TIFF image sequence, which was analysed with VGStudio Max 2.2. Segmentation of embryos from the adult, and of embryos from each other, was carried out by manually stepping through the image stacks and applying the 'draw' tool. Detection of embryos was performed based on dissection knowledge and checked in the 3D live view if necessary. Juveniles were given different colours when in the same bursae and, by setting the adult body to transparent, their posture and orientation could be studied *in situ*. Switching individuals 'on' and 'off' further revealed their position relative to each other. In order to render the volumes of the adult disc and each young, the 'adaptive rectangle' tool was applied on the most suitable 'iso' and 'depth' values. The volume of the adult torso (arms cut off closest to the disc) and all young was then carried out using the volume analysing function.

A standard desktop PC was used as operating system (CPU: Intel(R) Core(TM)i3 @2x 3.40GHz, Windows 7 Enterprise). Images and movies were created using the export function of VGStudio Max 2.2. Further image processing was performed using GIMP 2.8.

3. Results

3.1. Scans

Scans were of good quality and soft tissue was clearly visible. The best scan was achieved in a $(9\ \mu m)^3$ resolution trial of *Ophioderma wahlbergii*, which revealed good characteristics of the bursal wall. This thick organ spanned around the embryos and it seemed to be in close contact with the stomach (Figure 15). However, the field of view

was limited to one radius and due to the available time, priority was given to the analysis of the scan of the whole animal at $(30\ \mu m)^3$ resolution, where the quality was insufficient to accurately study the bursal wall. For potential future analyses the raw data of all scans is available at the Biological Science Department at the University of Cape Town.

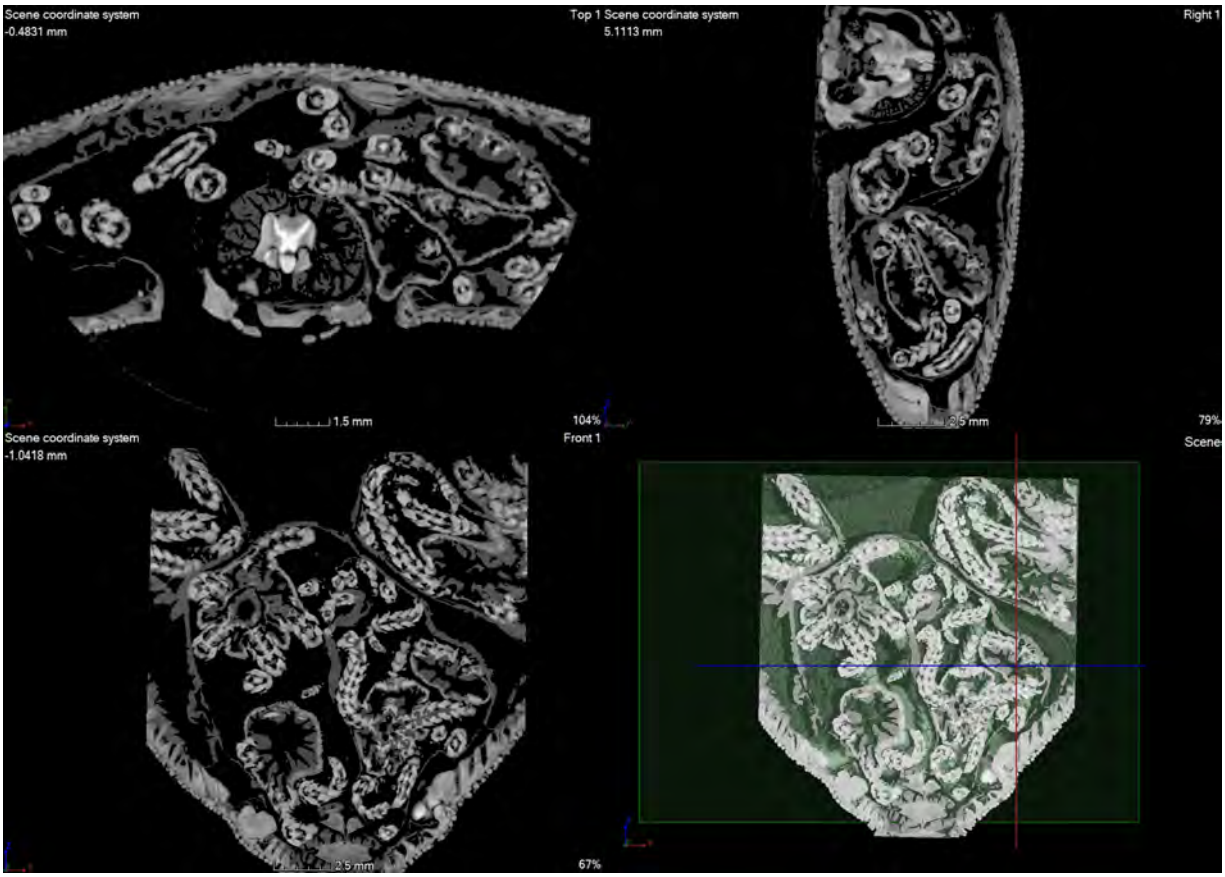

Figure 15. Radius of *Ophioderma wahlbergii* ($(9\ \mu m)^3$ resolution scan) in the desktop view of VGStudio Max 2.2. with all three dimensions and the rendered 3D model (cut open); only images exported using the export function.

3.2. Embryos and their position

The individual of *O. wahlbergii* was found to brood 13 large embryos in seven bursae. Of these, three bursae contained a single, large embryo, three contained two and one contained a total of four embryos (Figure 16, A-B). They were comparatively similar in size and the largest young measured about 6 mm in disc diameter.

All brooded young of *O. wahlbergii* were found to be orientated with the mouth facing to the top. In bursae with more than one embryo, the embryos lay upon another so that the mouth of the bottom one touched the back of its neighbouring sibling (Figure 16, B-C). The individual of *Amphipholis squamata* brooded a total of six embryos, with

two distinct size classes present (Figure 17, A, C). The larger ones had a disc diameter of about 1 mm and the smaller ones of about 0.25 mm. Not all had yet clearly developed discs. All embryos occupied separate bursae, although this was more difficult to judge than for *O. wahlbergii*.

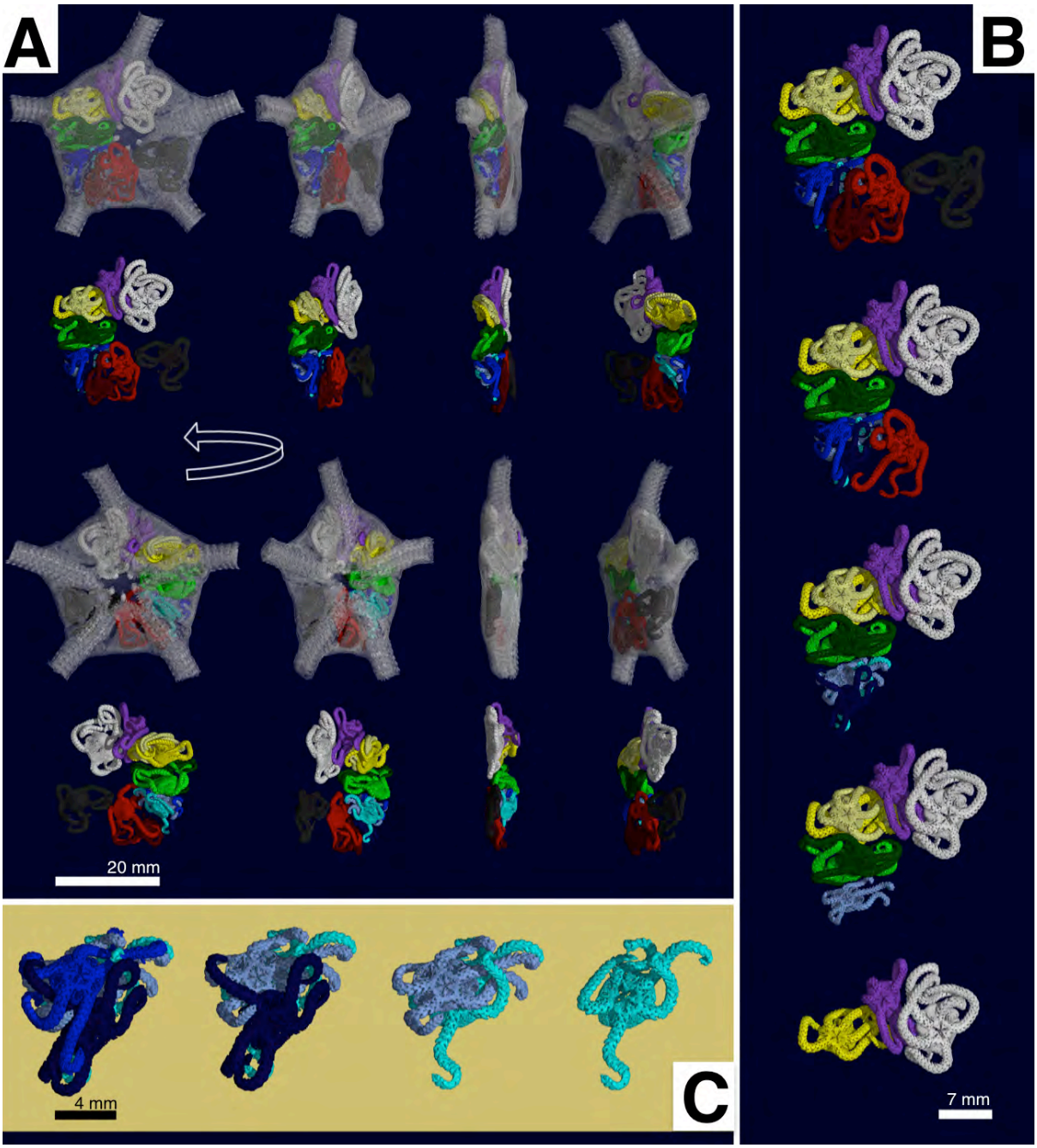

Figure 16. 3D model of *Ophioderma wahlbergii* containing 13 brooded young. **A** rotation of transparent adult disc and part of the arms with brooded young inside, first row, and brooded young without the adult disc, second row; arrow showing direction of rotation. **B**, **C** separation of brooded juveniles in detail.

Large and small brooded individuals of *A. squamata* alternated, so that all large and all small embryos lay in different interradii (Figure 17, C-D). Like in *O. wahlbergii* the embryos faced with their mouth towards the top. However, the orientation of the small ones remained unknown, because the mouth was not clearly formed yet, or not visible within the scans.

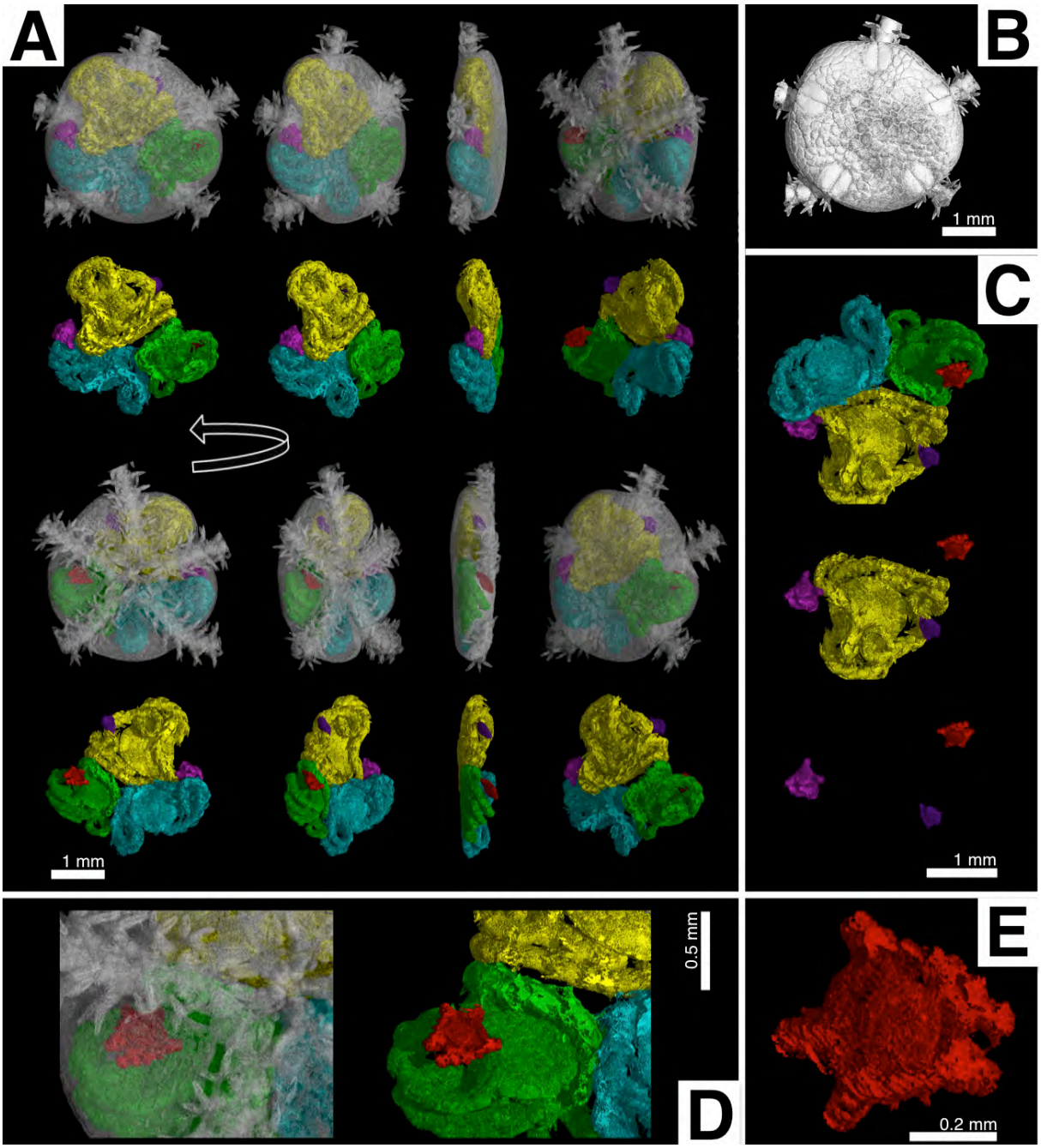

Figure 17. 3D model of *Amphipholis squamata* containing six brooded young. **A** rotation of transparent adult disc and part of the arms with brooded young inside, first row, and brooded young without the adult disc, second row; arrow showing direction of rotation. **B** disc in opaque mode. **C**, **D**, **E** separation of brooded juveniles in detail.

3.3. Volume analysis

The volume rendering showed that *A. squamata* dedicated 23.31% of its disc volume to brooding and *O. wahlbergii* 14.05%. In *A. squamata* the three large embryos made up 96% of the overall juvenile volume. Furthermore, the embryos of *O. wahlbergii* occupying a bursa on their own had a higher volume (Juvenile No. 1, 12 and 13, Table 5). In fact, the largest brooded young, No. 13 (118.23 mm^3), had a volume five times higher than the individual volume of the young inside the bursa containing four individuals. In comparison, the summarized juvenile volume per bursa was similar for all bursa that contained juveniles (Table 5, last column).

Table 5. Volume analysis of brooded young of *Ophioderma wahlbergii* and *Amphipholis squamata* (note that the colour refers to Figures 16 and 17).

	A. squamata		***O. wahlbergii***		
No. Juvenile	Volume (mm^3)	Colour/Bursa	Volume (mm^3)	Colour/Bursa	Volume per Bursae (mm^3)
1	0.26	green	72.18	black	72.18
2	0.01	pink	46.88	red1	92.76
3	0.37	turquoise	45.89	red2	
4	0.03	red	22.35	blue1	87.87
5	0.56	yellow	22.60	blue2	
6	0.01	purple	21.87	blue3	
7			21.05	blue4	
8			55.06	green1	101.54
9			46.48	green2	
10			37.39	yellow1	93.99
11			56.60	yellow2	
12			56.10	purple	56.10
13			118.83	grey	118.83
Sum Juvenile	1.23		623.27		
Disc Adult	5.06		4435.73		
Relation (%)	24.31		14.05		

4. Discussion

4.1. µCT scanning for brooding brittle stars

The quality of the data provides new insights into the brooding biology of ophiuroids, and the technique is applicable from small species <5 mm dd to large species >30 mm dd. Therefore, it covers the common size range of brittle stars and, because larger specimens are practicable to scan too, the technique is potentially suitable for all brooding echinoderms. In fact, the results reveal that in larger species even soft tissues,

like the bursal wall, are possible to visualise when applying finer voxel resolutions, such as $(9\ \mu m)^3$. Unfortunately, a higher resolution is accompanied by a narrower field of view, so that whole specimens are then difficult to scan. At least when unstained, in small species like *Amphipholis squamata*, the bursal walls or similar structures are too fine, even for high resolutions. A solution for the visualization of soft tissues is the use of density-changing staining treatments (Boistel et al. 2011). Contrast agents like iodine achieve considerable results when scanning invertebrates (Fernández et al. 2014). Because MRI is able to detect soft tissues, another advanced method is to overlay CT with MRI scans (Ziegler and Menze 2014). However, none of these soft-tissue techniques were necessary for the calcified skeleton of ophiuroids and, without any testing, this probably also applies to the other echinoderm classes.

Clearly, the strength of the method used is its non-destructive character and the completely intact specimen after the scan. All juveniles inside the brooding adult could be viewed in their actual position, and not, like in all studies conducted before, in an opened specimen with dissected disc and ruptured bursae. The only less destructive method possible would be to keep the animal alive, which was, for example, applied to measure gonadal indices of living starfish (Laforsch et al. 2012; Sigl et al. 2013). This would generally be conceivable for brooding echinoderms too, and it would allow for consecutive scans of the same individual to solve the problem of tracing embryonic growth (see Chapter 1). Moreover, the μCT scan method that was used is applicable to valuable museum specimens, such as type specimens. In the case of *Ophioderma wahlbergii*, the scanning of the holotype, based in Stockholm, would be of special interest. It does not show any signs of brooding from the outside (Stöhr 2014, pers comm). As discussed in Chapter 2, Branch and Branch (1992) were the first who discovered brooding in this species, but only 150 years after its original description. Does the first collected individual of *O. wahlbergii* contain brooded juveniles? Unfortunately, because of time limitations of this study, and problems of shipping the valuable specimen, this question remains to be answered.

4.3. Numbers, position and volume of juveniles

The numbers of the juveniles inside the scanned specimens is in general agreement with the known quantities of brooded young of both species (see Chapter 1 - 2). Moreover, both species are known as simultaneous brooders, bearing young of varying cohorts (Chapter 2, Hendler 1991). While this was confirmed for *A. squamata*,

the similar-sized young in *O. wahlbergii* are an exception, the reason probably being the sampling method in order to find the animal with the most juveniles.

It is suggested that brooded individuals of brittle stars, including the ones of *A. squamata*, take up nutrients from the mother by pressing their mouth against the bursal wall, and several species maintain this position in relation to the bursal wall (Walker and Lesser 1989; Byrne 1991; Byrne 1994; Hendler and Tran 2001). The scans clearly verify this embryonic behaviour, but its significance for the uptake of nutrients still needs to be demonstrated. Furthermore, using classical dissection techniques, Ludwig (1898), Mortensen (1920) and Hendler (1991) reported on brooded young of *A. squamata* taking a characteristic posture with the arms coiled over the disc, but this could not be verified in the *in situ* scan. This raises the question of whether the South African individuals can be considered the same as in other places. In fact, young of both species showed similar postures with the arms to the side, but it is probably the more limited space in the bursae of *A. squamata* that results in more compressed looking juveniles. In addition, the volume analyses confirmed the hypothesis that, overall, the smaller species devotes relatively more body space to brooding. However, the 10% difference of relative volume dedicated to brooding would need to be reinforced by a larger sample size. Over all, the brooding strategies of the two very different, and only distantly related species can be considered fairly similar.

4.2. 3D visualizations

Gillespie and McClintock (2007) discussed how modern techniques could address important questions about brooding echinoderms, but they omitted 3D imaging. However, this study has shown that 3D imaging and derived measurements can add to the historical perspective on brooding echinoderms, and the possibilities of such scans will not be exhausted for a long time to come. Movies of 3D animations, but also interactive 3D PDFs, where the observer rotates the object to decide on the perspective, serve to understand internal structures (Murienne et al. 2008), and also ecological adaptations such as brooding. Movies of the scans of this study can be found on the compact disc attached to this thesis, retrieved from the Biological Sciences Department of the University of Cape Town, or downloaded under http://landschoff.net/blog/wordpress/wp-content/uploads/sites/3/2014/07/Ophioderma.mp4 or under http://www.youtube.com/watch?v=-d0kHTNmkkk. The application of these animations must also be seen in the context of teaching, as well as communicating science to a wider audience. In

the end, any 3D model can also be magnified, printed in 3D and, for example, be displayed in museums. Because of the complex models (transparent and various colours), this was not feasible for this project. However, the substantial progress of 3D printers will promote the availability of 3D prints of digital imaging techniques such as μCT scans.

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